IDENTIFICATION OF BACTERIA ISOLATED FROM MALT, WITH THE EMPHASIS ON LACTIC ACID BACTERIA AND THEIR INFLUENCE ON BREWER'S YEAST

by

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own and has not previously, in its entire or in part, been submitted at any university for a degree.

Clifford Booysen

SUMMARY

Changes in the bacterial population throughout the malting process of two barley cultivars, i.e. Clipper (local cultivar) and Prisma (imported cultivar), malted at Southern Associated Maltsters (SAM), Caledon, South Africa, were studied. Samples were taken from four individual runs of each cultivar at ten different stages, i.e. dry barley before steep, water from the first steep water-stand, barley after draining the first steep, water from the second steep water-stand, barley from the second steep water-stand, barley after draining of the second steep, barley from the first, second and third days of germination in the germination vessels (GV), and malt after kilning. Emphasis was placed on the taxonomy and composition of the lactic acid bacteria (LAB) isolated from the ten different phases.

The LAB were identified to species level by using numerical analysis of total soluble cell protein patterns, RAPD-PCR banding patterns and 16S rRNA sequencing. The Gram-negative bacteria were identified to genus level by using the API 20E system and included *Citrobacter* spp., *Enterobacter* spp., *Pantoea* spp., *Proteus* spp., *Seratia* spp., *Kluyvera* spp., *Klebsiella* spp., *Vibrio* spp. and *Escherichia coli*. The number of viable bacteria throughout the malting process of the two cultivars did not differ significantly, although the LAB counts in the barley before steep and on the kilned malt were higher in Prisma than in Clipper. *Leuconostoc argentinum*, *Leuconostoc lactis* and *Weissella confusa* were the most predominant in both cultivars. A few strains of *Weissella paramesenteroides*, *Lactobacillus casei*, *Lactococcus lactis* and *Lactobacillus rhamnosus* were also isolated. *Lb. casei* and *Lb. rhamnosus* were not isolated from the Prisma cultivar, whilst *W. paramesenteroides* and *Lc. lactis* were absent in the Clipper cultivar. Kilned malt of the Clipper cultivar contained predominantly *Le. argentinum*, whereas the Prisma cultivar contained mainly *Lc. lactis*.

The effect of these bacteria on the fermenting ability of the brewer's yeast *Saccharomyces cerevisiae* SAB 05, was also studied. Fermentations were conducted in wort prepared from Clipper and Prisma malt. Yeast in combination with the different bacteria were used in the fermentation studies. Wort with only yeast was used as control. Emphasis was placed on the effect the bacteria has on the gravity, pH, yeast- and bacterial- counts and the different volatile aroma compounds

produced throughout the fermentations.

The presence of LAB and Gram-negative bacteria had no effect on the yeast to reduce the gravity of the fermenting wort, whilst the LAB caused a decrease in the pH of the fermentations in both Clipper and Prisma wort. The cell numbers of the Gram-negative bacteria decreased throughout the fermentations, whilst the LAB cell numbers remained constant. Comparisons could be drawn between the volatile aroma compounds produced in the control fermentation and fermentations with yeast and Gram-negative bacteria, yeast and *Lactobacillus* spp. and yeast and *Weissella* spp. *Leuconostoc* spp. had a much greater influence on the aromatic composition of fermented malt, with much more clear variations between Prisma and Clipper. No major differences were recorded in the aroma profiles of Prisma and Clipper malt fermented in the presence and absence of *Lactococcus* spp. The Gram-negative bacteria had no significant effect on the volatile aroma compounds produced by the yeast, whilst the LAB had a definite effect on aroma composition in both cultivars. The levels of four of the five principle aroma compounds, present in beer, were in the acceptable concentration range on the final day of fermentation. The compounds with the highest concentrations were iso-amyl alcohol, acetic acid and acetoin, with acetic acid being present in the highest concentration in all the fermentations.

OPSOMMING

Veranderinge in die bakteriese populasie van die gars kultivars, Clipper (plaaslik) en Prisma (ingevoer), vermout by Southern Associated Maltsters (SAM), Caledon, Suid Afrika, is ondersoek. Monsters is van vier individuele lopies van elke kultivar en tydens tien verskillende fases van die vermoutingsproses geneem. Die tien verskillende stadia het die volgende ingesluit: Droë gars voor benatting, water van die eerste benattingsfase, gars nadat water van die eerste benattingsfase gedreineer is, water van die tweede benattingsfase, gars van die tweede benattingsfase, gars na die dreinering van water in die tweede benattings fase, gars na die eerste, tweede en derde dag van ontkieming binne die ontkiemingstenke, en mout na droging. Klem is geplaas op die taksonomie en samestelling van melksuurbakterieë (MSB) wat tydens die tien verskillende fases geïsoleer is.

Die MSB is tot spesievlak geïdentifiseer deur gebruik te maak van numeriese analise van totale oplosbare selproteïen bandpatrone, RAPD-PKR bandpatrone en 16S rRNA volgorde-bepaling. Gram-negatiewe bakterieë is tot op genusvlak geïdentifiseer deur gebruik te maak van die API 20E toetssisteem. Spesies van die genera *Citrobacter*, *Enterobacter*, *Pantoea*, *Proteus*, *Seratia*, *Kluyvera*, *Klebsiella*, *Vibrio* asook *Escherichia coli* is geïdentifiseer. Tydens die vermoutingsproses van die twee kultivars is geen beduidende verskille in die lewensvatbare bakterietellings gevind nie, alhoewel die MSB-tellings in die gars voor benatting en mout na droging in Prisma hoër was as in Clipper. *Leuconostoc argentinum*, *Leuconostoc lactis* en *Weissella confusa* het die meeste voorgekom in beide kultivars. Kleiner hoeveelhede van *Weissella paramesenteroides*, *Lactobacillus casei*, *Lactococcus lactis* en *Lactobacillus rhamnosus* is ook geïsoleer. *Lb. casei* en *Lb. rhamnosus* het nie in die Prisma-kultivar voorgekom nie, terwyl *W. paramesenteroides* en *Lc. lactis* nie in die Clipper-kultivar teenwoordig was nie. *Le. argentinum* het meestal in die gedroogde mout van die Clipper-kultivar voorgekom, terwyl *Lc. lactis* meestal in die Prisma-kultivar waargeneem is.

Die effek van hierdie bakterieë op die fermentasievermoë van die brouersgis *Saccharomyces cerevisiae* SAB 05 is ook bestudeer. Die fermentasies is in Clipper- en Prisma- wort gedoen. Vir die fermentasiestudies is gis in kombinasie met verskillende bakterieë gebruik. Wort met slegs gis het as kontrole gedien. Klem is geplaas op die effek van die bakterieë op die digtheid, pH, gis- en bakterie-tellings en die verskillende vlugtige komponente wat tydens die fermentasies geproduseer is. Die teenwoordigheid van MSB en Gram-negatiewe bakterieë het geen effek gehad op die vermoë van

die gis om die digtheid van die gefermenteerde wort te verlaag nie. Die MSB het wel 'n verlaging van die pH in beide Clipper- en Prisma- wort teweeggebring. Tydens die fermentasie het die Gram-negatiewe bakterietellings verminder, terwyl die MSB-tellings konstant gebly het. 'n Verband is gevind tussen vlugtige komponente geproduseer in die kontrole-fermentasie en fermentasies met gis en Gram-negatiewe bakterieë, gis en *Lactobacillus* spp. en gis en *Weissella* spp. *Leuconostoc* spp. het groter veskille in die samestelling van die gefermenteerde wort teweeg gebring met duidelike verskille tussen Clipper en Prisma. Die teenwoordigheid van *Lactococcus* spp. het nie groot verskille in die samestelling van die gefermenteerde wort getoon nie. Op die laaste dag van die fermentasies was die vlakke van vier uit die vyf belangrikste vlugtige aroma komponente wat in bier voorkom in die kontrole fermentasies in aanvaarbare konsentrasies teenwoordig. Die Gram-negatiewe bakterieë het geen beduidende invloed gehad op die vlugtige aroma komponente wat deur die gis geproduseer is nie, terwyl die MSB 'n besliste effek in die aroma-samestelling van beide die kultivars gehad het. Die komponente met die hoogste konsentrasies was, isoamiel-alkohol, asynsuur en asetoin. Asynsuur was in al die fermentasies in die hoogste konsentrasie teenwoordig.

BIOGRAPHICAL SKETCH

Clifford Booysen was born on the 6th of February 1976 in Malmesbury. He matriculated from the Atlantis Secondary School in 1993 and thereafter enrolled at the University of Stellenbosch. In 1998 he obtained his B.Sc. Agric. degree, with Biochemistry, Genetics and Microbiology as majors.

PREFACE

The Literature review includes an overview of the malting and brewing processes, morphology of the barley kernel and the changes, morphological and microbiological, that occurs during malting. Taxonomic methods used to classify lactic acid bacteria (LAB), with special emphasis on species found in malting and brewing, and their effect on brewers yeast are discussed.

The paper, "Isolation, identification and changes in the composition of lactic acid bacteria during the malting of two different barley cultivars" has been accepted for publication in International Journal of Food Microbiology.

The second paper, "Effect of bacteria on the fermenting ability of brewer's yeast," has been written according to the style of the Journal of the Institute of Brewing.

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CONTENTS

1. INTRODUCTION

INTRODUCTION

There are two main processes involved in the production of malt, namely germination of barley and spontaneous fermentation. The fermentation processes and environmental conditions in the malting house affect malt properties significantly in various ways. Most studies on the proliferation of lactic acid bacteria (LAB) during the malting process were conducted with laboratory-scale malting trials. Little is known about the activity of these microorganisms in commercial malting systems, probably because of the difficulties encountered in determining the specific effects these bacteria have on malting and brewing.

Unprocessed barley contains a significant number of LAB (Douglas and Flannigan 1988; Peters *et al.* 1988; Noots *et al.* 1998). Although some of these bacteria wash out during the steeping process, cell counts increase significantly during steeping and remain high during germination. Kilning causes a reduction in viable cell counts of at least 98%. In unprocessed barley, the LAB population is roughly divided into *Leuconostoc mesenteroides* (50%), *Lactococcus lactis* (25%) and *Lactobacillus* spp. (25%). The latter species include *Lactobacillus delbrueckii* subsp. *delbrueckii, Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactobacillus brevis* and *Lactobacillus buchneri* (Douglas and Flannigan 1988; Peters *et al.* 1988; Noots *et al.* 1998). These lactobacilli are predominantly found in germinated and steeped barley (Peters *et al.* 1988). The *Leuconostoc* spp., on the other hand, are more prevalent during steeping (Peters *et al.* 1988; Noots *et al.* 1998). These bacteria and their metabolic products have a negative influence on the malting and brewing processes and the quality of beer.

Interference with barley respiration, secretion of enzymes, hormones, toxins and acids, which may affect the physiological processes of germinating barley, has been studied. Organic acids, in particular lactic acid produced by LAB at different stages of the malting process, acidifies the malt bed and influences germination. High LAB counts on malt may influence brewhouse performance, e.g. the wort nitrogen content, pH, colour of wort and, ultimately, yeast fermentation. Certain strains of *Leuconostoc* spp. produce bacterial capsules and slime which interferes with mash filtration. *Lactobacillus brevis* causes ropiness and super-attenuation. Certain strains of *Lactococcus* spp. cause sarcinae sickness, whereas *Lactobacillus* and *Lactococcus* spp. increases the turbidity of beer (Ziola *et al.* 1992).

Not all LAB are detrimental to the brewing process. Some strains, e.g. *Lb. plantarum, Lb. delbrueckii* and *Lb. acidophilus* are used as starter cultures in malting to restrict the growth of *Fusarium* spp., Gram-negative and Gram-positive bacteria. Inoculation with LAB starter cultures also leads to improvements in the physical and chemical quality of malt and minimises the chances of mash filtration difficulties (Haikara and Home 1991).

To understand the microbial interaction in barley grain, a detailed study of the microflora and changes in the microbial ecology during barley growth, storage and malting needs to be done. The impact of these microorganisms, including LAB, on malt quality has to be investigated. In this study the different stages of the malting and brewing processes are reviewed. The microorganisms present, especially the LAB which occur during malt production was identified. A preliminary study on the effect these microorganisms have on the fermenting ability of brewer's yeast has been conducted.

REFERENCES

Douglas, P.E., Flannigan, B. (1988) A microbial evolution of barley malt production. *Journal of the Institute of Brew*ing **94**, 85-88.

Haikara, A., Home, S. (1991) Mash filtration difficulties caused by split barley kernels: A microbiological problem. *Proceedings of the European Brewery Convention Congress*, Lisbon, 537-546.

Noots, I., Delcour, J.A., Michiels, W. (1998) From field barley to malt: detection and specification of microbial activity for quality aspects. *Critical Reviews in Microbiol*ogy **25**, 121-253.

Peters, H.I., Flannigan, B., Austin, B. (1988) Quantitative and qualitative studies of the microflora of barley malt production. *Journal of Applied Bacteriol*ogy **65**, 279-297.

Ziola, B., Whiting, M., Crichlow, M. and Ingledew, W.M. (1992) Detection of *Pediococcus* spp. in brewing yeast by rapid immunoassay. *Applied and Environmental Microbiology,* **58**, 713-716.

2. OVERVIEW OF THE MALTING AND BREWING PROCESSES AND CHANGES THAT OCCUR IN THE BARLEY KERNEL DURING MALTING

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INTRODUCTION

Barley (*Hordeum vulgare* L.) is a member of the *Graminae* family which contains all the grasses, including wheat and sorghum. Barley is not only an important feed crop, but also the predominant raw material used in the production of malt for brewing purposes (Linko *et al.* 1998). Thousands of years ago beer was produced without any knowledge of microorganisms or enzymes. Despite being referred to as a typical example of traditional biotechnology, beer brewing is on the forefront of biotechnological development (Haikara and Home 1991; Linko *et al.* 1998).

Although the physiology of the barley kernel is of the utmost importance in malt and brewing quality, the impact of the natural microbial population on malt quality throughout the production chain cannot be ignored. Many studies have shown that microbial processes influence the malt characteristics in different ways (Bol *et al.* 1985; Biovin *et al.* 1996; Bellimov *et al.* 1998). To understand the microbial metabolic activity during malt production and its interaction with barley grain, the microbial changes that occur during barley growth, storage and during the malting process need to be understood.

During growth in the field barley becomes infested with a variety of microorganisms. The barley is normally harvested when the moisture level is between 16 and 20% (m/v). The barley is then artificialy dried or left in the field to achieve a moisture content of ca. 12% (Noots *et al.* 1998).

The humidity of the grain seeds throughout the malting process changes from a high water content in steep and during germination and a low water content after kilning. The microbial activity in the barley kernels changes as the humidity decreases (Vaag 1991; Eagles *et al.* 1995). These changes also bring about changes in enzyme levels, sugars, amino acids and other metabolic compounds that may develop during germination (Peters *et al.* 1988; Gorg 1992). The bacteria and fungi can contribute to higher enzyme levels with the different enzymes they secrete. Infections of malt by some fungi, e.g. *Aspergillus* and *Fusarium* spp., and bacteria are known to cause off flavours and gushing in beer (Peters *et al.* 1988; Gorg 1992).

OVERVIEW OF THE MALTING PROCESS

The South African Barley Story

Barley is the primary essential raw material in the manufacturing of beer. In South Africa barley is grown predominantly in the South Western Cape, in a region called the Ruens (Fig.1), which, with its Mediterranean-type climate, is well suited for the cultivation of winter cereals. A good distribution of rainfall during the growing season, followed by a warm, dry harvest, is essential for both good barley yield and malting quality. The annual rainfall in this region is reasonably reliable and varies from 380 mm to 520 mm, 65 percent of which falls in winter.

The soil types and climate of the Ruens demand specialised cultivation techniques founded on a system of crop rotation. This rotational system is essential to suppress root diseases and regulate the nitrogen content of the soil. Barley absorbs nitrogen from the soil, which is converted to protein in the grain. Nitrogen levels in the crop are crucial for quality and have to fall within a narrow range for the crop to qualify as malting barley (Jenkyn *et al.* 1992).

The barley seed and seedbed are prepared carefully before sowing in May. To maximise yield and quality, fertiliser is applied during the sowing and later as top dressing, at specific growth stages, if required. Spraying the fields with pesticides, usually from the air, controls fungi and other pests. In November the barley is usually harvested by a traditional method known as swathing. This process lasts about ten days and involves cutting the crop and leaving it in the field to dry. Thereafter, the barley is threshed by a combine harvester and delivered to the farmers' co-operatives where it is sampled, classified and graded. The grain is usually handled in bulk and stored in concrete silos. To preserve the quality of the barley, the silos are aerated with cool, fresh air and the temperature of the grain mass constantly monitored.

The main cultivar grown in the Ruens since 1975 is Clipper, although new cultivars with different quality characteristics, which are crucial elements in maintaining a viable barley industry, are also grown in this region. Despite the large amounts of barley being produced in the Western Cape, South Africa regularly imports barley from Europe and Australia.

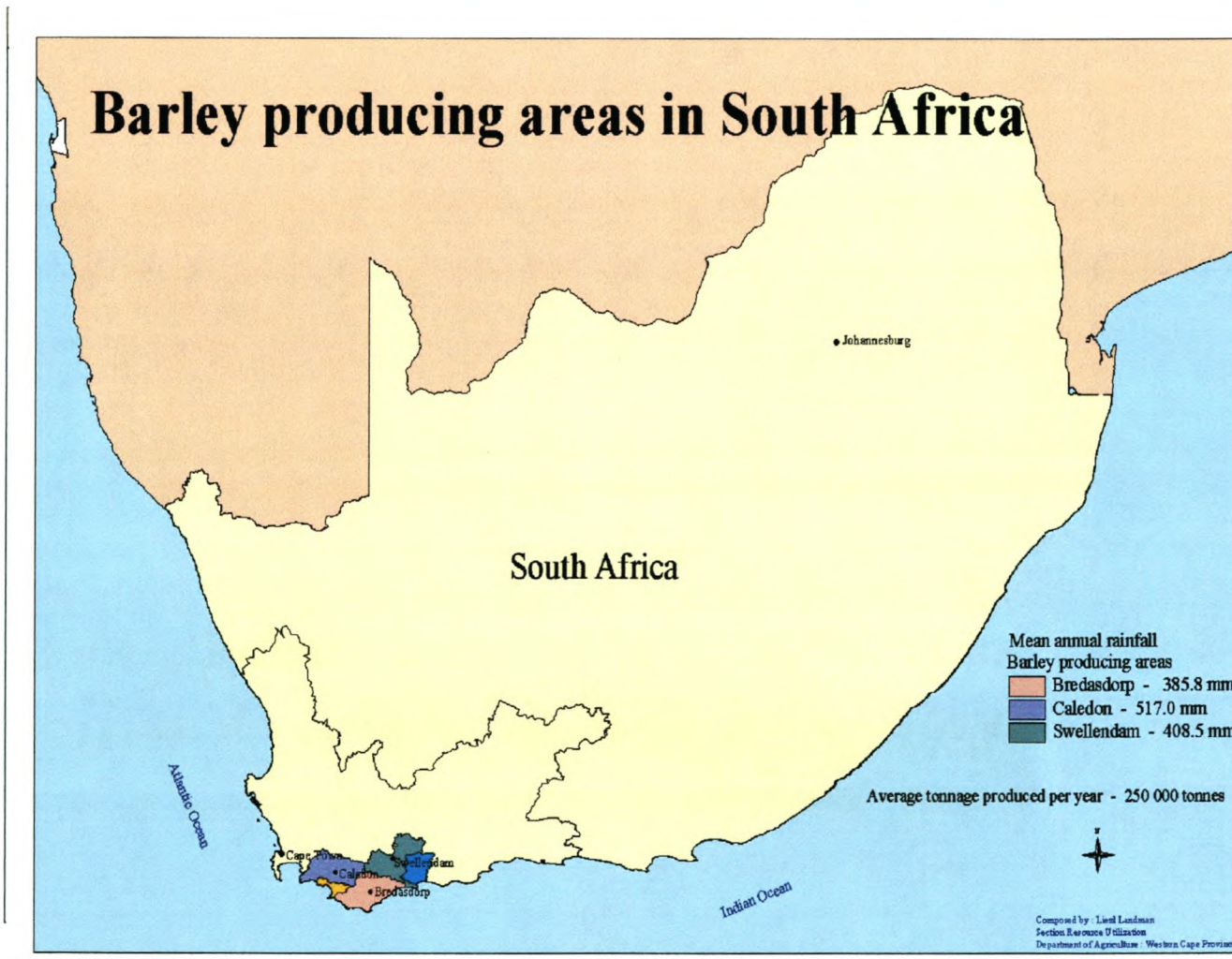


Fig. 1. Map of the barley producing region (Ruens) in South Africa.

MORPHOLOGY OF THE BARLEY KERNEL

There are a vast number of Barley varieties all over the world. In South Africa two barley varieties, six-row and two-row barley, were planted until 1977/78. The six-row barley has since been discontinued. Different barley varieties also come from different regions, e.g. Franklin, Schooner and Arapiles from Australia, Alexis from Continental Europe, and Oxbow and Harrington from Canada.

If viewed from the top, six-row barley has six rows of kernels around a central "axis", with three kernels per node on alternative sides. Two-rowed barley has only one kernel developed on opposite sides of the axis, resulting in two rows of kernels. Furthermore, two-row barley is more plump than six-row barley and the kernels appear twisted and slender.

A longitudinal illustration of the mature barley kernel is shown in Fig. 2a. Seven main areas can be identified i.e. the husk, pericarp, testa, endosperm, embryo, aleurone layer and the scutellum (Fig. 2b).

The outer protective silica coating of the kernel, i.e. the husk (65% silica) is tightly adhered to the internal part of the kernel, or it may be loose, depending on the barley variety. The layer is normally thicker in the embryo area where it serves as a protective shield. The layer also serves as a filter during mash filtration in the “lauter tun”.

The pericarp and testa are immediately beneath the husk and are closely adhered to each other. This semi-permeable membrane covers the entire kernel. The testa effectively separates the exterior of the barley kernel from the interior of the grain and is only premeable to water, but prevents the passage of acids, alkalis, dissolved salts and most ionized substances into the kernel.

The Endosperm is covered by the aleurone layer and is the largest part of the barley kernel. The endosperm acts as food reserve for the growing barley plant and consists of 80% starch. The cells are packed with large and small starch granules embedded in a protein matrix.

The aleurone layer is the layer where all enzymes are synthesised. Each cell has its own nucleus, cytoplasm, aleurone granules, protoplasm and lipids.

The Embryo produces the rootlets and the shoot (ascospire; coleoptile) of the developing plant. It consists of the embryonic leaves, primordia and a leaf sheath. The scutellum is a flattened body of cells between the embryo and endosperm.

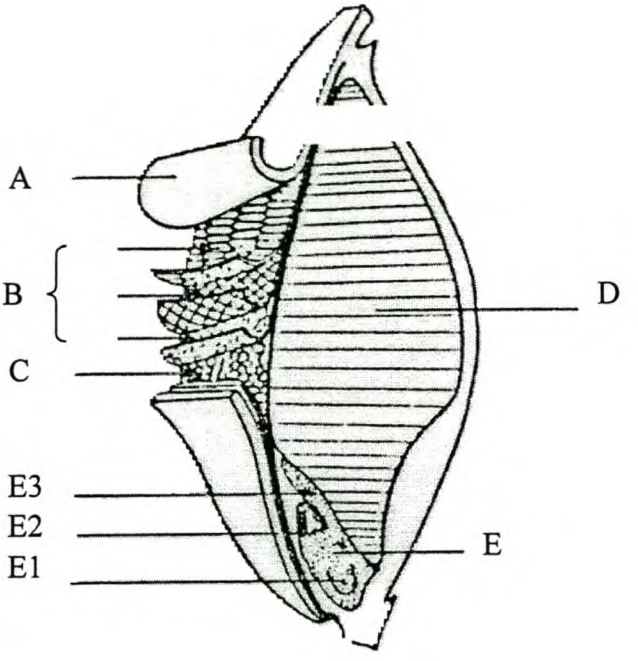
Chemical analysis of the mature barley grain revealed the following composition (average figures): Moisture content between 12-17%; dry matter ca. 65% starch, located in the starch granules of the endosperm; 10-12% protein, located in the husks, in the aleurone cells, the embryo and between the starch granules of the endosperm; 6% cellulose, located mainly in the husk; 9% pentosans, including hemicellulose, located mainly in the cell walls; 3% lipids, located in the embryo and in the aleurone cells; and other inorganic matter, tannins, coloured compounds, vitamins and enzymes.

The Malting Process

Malting is the artificial induction and controlled germination of grain, which is thereby transformed into so-called malt. The terms malt and malting is applicable to any germinated grain. The grain that is usually malted is barley. The main aim of malting barley is to obtain certain physiological and chemical changes within the grain. These changes are desired in beer brewing, production of wheat or corn flakes, and other food products and beverages, e.g. distilled spirits such as whisky (Linko *et al.* 1998; Noots *et al.* 1998).

The malting process involves steeping of the barley in water, followed by germination and kilning or drying of the germinated barley. Cultivation, harvesting and storage of the cereal are considered part of the production chain (Fig. 3). The microbiological composition of the barley kernel is to a large extent influenced by the conditions prior to the actual malting process (Linko *et al.* 1998; Noots *et al.* 1998).

a.



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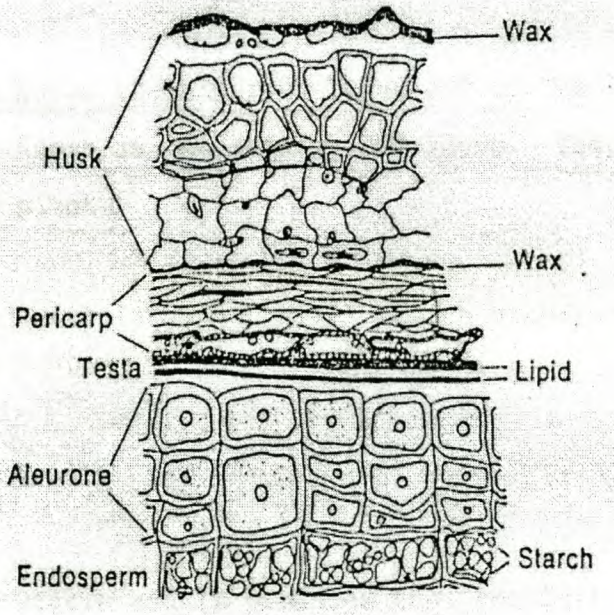

Fig. 2. (a) Structure of the barley kernel. (A) Husk, (B) Pericarp and Testa, (C) Aleurone Layer, (D) Endosperm, (E) Embryo (Germ), with (E1) Rootlets, (E2) Ascospire and (E3) Scutellum. (b) Transverse section of the outer layers of the barley kernel. (from: Noots *et al.* 1998)

Quality is of the utmost importance and receives a great deal of attention throughout the process. With the barley still in the field, it is closely monitored from the cultivation stage through to harvesting and delivery to the farmers' co-operatives. A good barley is characterised as having plump kernels even in size (at least 80% of the kernels larger than 2.5mm), a nitrogen content of between 1.35 and 1.95% and a high potential activity of α-amylase, β-amylase, protease and β-glucanase (Jenkyn *et al.* 1992). The protein level of the kernels has to be between 10.5 and 11.5% and should produce malt with a high extract- and diastatic- power (Eagles *et al.* 1995). The barley should also be free of any insect infestation and moulds such as *Fusaruim* spp. and *Aspergillus* spp. (Bol *et al.* 1985; Eagles *et al.* 1995).

During storage of the barley in silos, the condition of the barley is constantly monitored to ensure that no deterioration takes place. The temperature of the stored barley is kept low (approx. 14°C) by blowing fresh, cool air through the silos. This prevents the kernels from loosing germination vigour and discourage insect infestation (Bol *et al.* 1985). Before actual processing, a representative sample of the barley is taken from every silo and processed on a micro scale. This micro-malting process provides the maltster with information to predict the quality of the malt and to select for barley with other characteristics. This is done to ensure that the malt conforms to certain specifications.

Pre-cleaning of the barley on special machines, which removes dirt, dust, fractured grains and small barley kernels, is the first step of the malting process and ensures that good quality barley is malted. When barley for example contains more than 2% damaged grains, difficulty in filtering during the brewing process is experienced. This is not due to a lack of modification, but to the production of extracellular polysaccharides by *Leuconostoc* spp., *Flavobacterium* spp. and *Pseudomonas* spp., which grow on damaged kernels (Folstad and Christensen 1962; Haikara and Home 1991).

Steeping: Steeping is the immersion of barley (ca. 500 t) in fresh, cold water to initiate germination (Peters *et al.* 1988; Linko *et al* 1998; Noots *et al.* 1998). This is similar to what happens to barley seed in soil. Steeping is done in a large sement tank (Fig. 4) where repeated water immersions alternate with dry rest periods. The tank is aerated by blowing cool moist air through the grain bed to encourage even moisture uptake by the kernels (Peters *et al.* 1988;

Linko *et al* 1998). The steeping process usually takes 40 to 48h. To produce beer malt in South Africa the moisture content of the barley is increased from 12% to 45% during a period of two days. The temperature, CO_2 and O_2 levels during steeping are accurately controlled. At the end of steeping when the first shoot (chit) of the germinating seeds are visible, the barley is transfered over to germination vessels.

Germination: Germination is a 3 to 4 day stage in the malting process, during which the barley seed is allowed to grow. This takes place in a specially designed vessel, which enables the maltster to guide the process. The grain then undergoes external and internal changes. The rootlets break through the embryo end of the grain and the sprout (ascospire) grows from the embryo under the husk along the dorsal side (Noots *et al.* 1998). Several morphological and biochemical changes are also taking place inside the barley seed. The biochemical changes, e.g. production of hormones that stimulate the production of enzymes by the aleurone layer and interaction of these enzymes with the starch and amino acid residues is called modification (Linko *et al.* 1998). The heat and CO_2 liberated by respiration are removed by blowing chilled, humidified air through the germinating barley, now called green malt. By doing this, O_2 is supplied and the temperature is kept between 14 and 18°C to maintain the humidity of the grain bed.

The growing roots are prevented from "mating" with each other by turners that rotate the malt every 12h and aerate the green malt bed (Fig. 5). This also ensures homogeneous processing conditions. After 3 to 4 days the germination process is stopped by transferring the green malt to the kilning vessels.

Kilning : The kilning process may be conducted in the germination vessel, or in a separate vessel specially designed for the purpose. During this stage water is removed from the green malt and all enzymatic reactions halted to stabilise the malt so that it can be stored without deterioration. The malt is also slightly roasted to produce a specific colour and flavour (Linko *et al.* 1998; Noots *et al.* 1998).

The combination of high grain moisture and high temperature destroys the enzymes which develop during germination. Fresh heated air (50-60°C) is blown through the green malt bed to

remove most of the moisture and preserve the enzymes required for starch and protein hydrolysis in the brewing process (Linko *et al.* 1998; Noots *et al.* 1998). When the moisture content of the green malt is approx. 10%, the kilning temperature is increased to between 80°C and 90°C to caramelise the sugars in the kernel and produce malt with a characteristic colour and flavour and a moisture content of 4%, m/v (Linko *et al.* 1998). This conversion of barley to malt is termed "modification", an important characteristic which renders beer its unique colour and flavour (Hough and Kleyn 1971; Linko *et al.* 1998). After kilning the dried rootlets are removed and sold to farmers as animal feed. The final product (malt) is then stored in silos at the malting plant and later blended with other malt to produce the preferred blend for each specific beer.

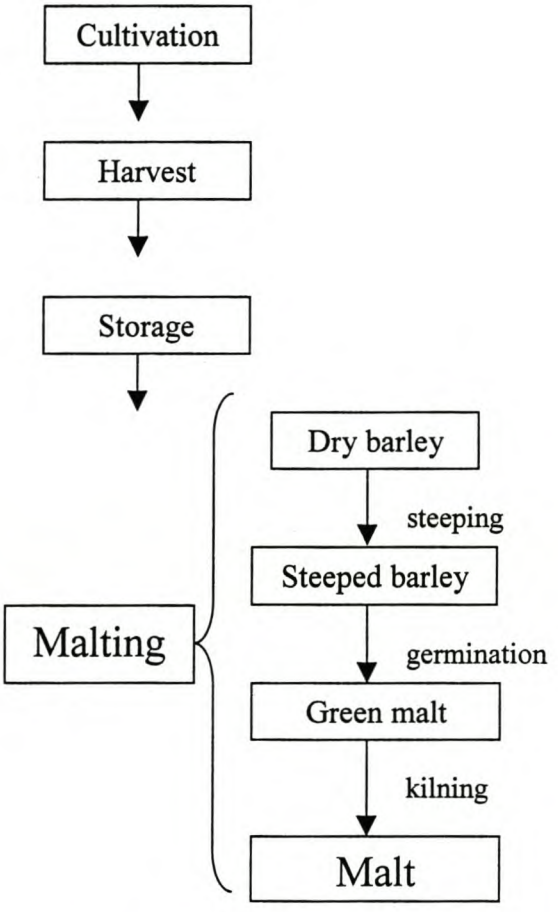

Fig. 3. Simplified diagram of the malt production chain.

During the malting process, the maltster carefully monitors the barley's progress. Traditional skills, such as "rubbing" and sensing of the fresh cucumber-like aroma, are supplemented by sophisticated scientific analyses. On completion of the malting stage, a sample is drawn from

each batch and subjected to analyses. The fermentable extract, known as sweet wort, is analysed for critical parameters as specified by the brewers. On the basis of these analyses the batches of malt are blended to provide consistency of quality. The blended malt is analysed again before it is despatched to the breweries.

The malting process is a delicate process and needs to be well controlled. As the barley seed germinates it utilise some available nutrients and enzymes. It is thus important for the maltster to control the malting process so that the seed uses a limited concentration of these nutrients. This is done by carefully controlling the temperature, time and water content during the malting process (Noots *et al.* 1998).

OVERVIEW OF THE BREWING PROCESS

The conventional brewing process, outlined in Fig. 6, illustrates the production of so-called lager beer with bottom fermenting yeast. Many variations of the brewing process exist, depending on the specific type of beer. Ale and stout are produced with top fermenting yeast and the process differs from lager beer.

Beer fermentation is divided into four stages (Fig. 6):

After the malt arrives at the breweries it is milled or grounded and mixed with water. The temperature of this mixture (mash) is slowly increased for extraction and hydrolysis of the malt constituents, which is primarily starch, but also components such as proteins and β-glucan. By carefully controlling these temperatures, the brewer produces an extract containing the correct proportions of malt sugar, protein, amino acids, mineral salts and B-group vitamins to produce beer with a balanced flavour.

The enzymes hydrolyse the starch and proteins to produce fermentable sugars and amino acids. The non-soluble components are separated either in an automatic mash filter, or in a so-called "lauter tun", in which the non-soluble constituents, especially the husks, form a filter layer. At this point other cereals, sugars and hops or hop extracts are added to the filtrate (wort).

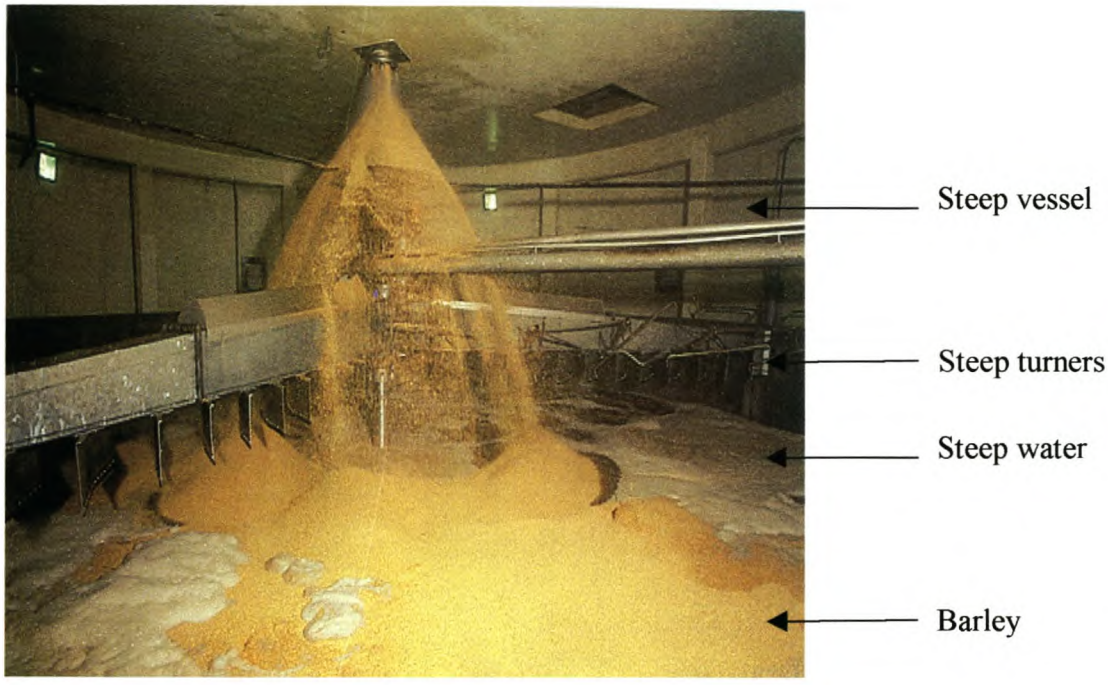

Fig. 4. Barley mixed with water in a steeping vessel (photographed at SAM, Caledon).

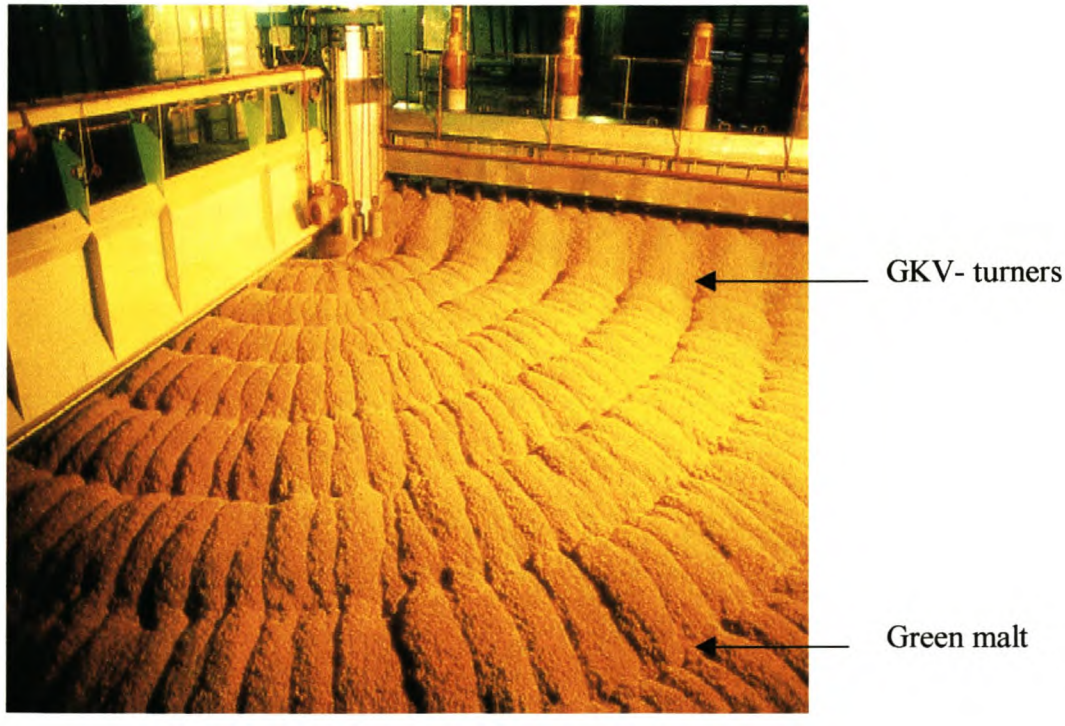

Fig. 5. Barley in a germination vessel (photographed at SAM, Caledon).

Hops render beer its characteristic bitter and aromatic taste (Smith and Simpson 1992). The wort is then boiled to inactivate any microbial growth (Hough and Kleyn 1971; Linko *et al.* 1998). At these high temperatures the proteins and other undesirable substances react with each other and precipitate to the bottom of the tank (coagulation). The precipitate is removed in a wort cyclon (centrifuge) and finally cooled.

After cooling, the wort is inoculated with yeast. Fermentation in most cases is divided into primary or main fermentation and maturation or secondary fermentation. The primary fermentation, which is the alcoholic fermentation, takes seven days and the secondary fermentation two weeks (Linko *et al.* 1998). Most of the flavour compounds are produced during the main fermentation. Relatively few changes occur during maturation (secondary fermentation, lagering). The fermented wort (beer) is filtered, stabilised, pasteurised and bottled.

Diacetyl, characterised by its butter-like aroma, forms as a by-product during primary fermentation and is linked to amino acid metabolism (Linko *et al.* 1998). The concentration of diacetyl in lager beer has to be below the taste threshold, i.e. between 0.1 and 0.2 mg/l. At the end of the main fermentation the concentration of diacetyl is usually above the taste threshold, but slowly decreases during maturation to produce a matured product with acceptable diacetyl levels (Linko *et al.* 1998). The formation of diacetyl from α-acetolactate is a non-enzymatic reaction. The subsequent reduction of diacetyl to acetoin and further to 2,3-butanediol is enzymatic (Fig. 7). The enzymatic conversion from α-acetolactate to acetoin (Fig. 7), is not performed by brewer's yeast, since they lack the enzyme α-acetolactate decarboxylase (α-ALDC). Several *Lactobacillus* spp., *Leuconostoc* spp., *Lactococcus* spp. and certain Gram-negative bacteria produce α-ALDC. The taste threshold for acetoin is much higher than that for diacetyl. Thus, by proceeding straight to acetoin the off-flavour problem is solved (Linko *et al.* 1998).

The enzyme α-ALDC can be produced separately and added during primary fermentation. This enzyme is now commercially available. Another option is to clone the gene responsible for the production of α-acetolactate decarboxylase into brewer's yeast.

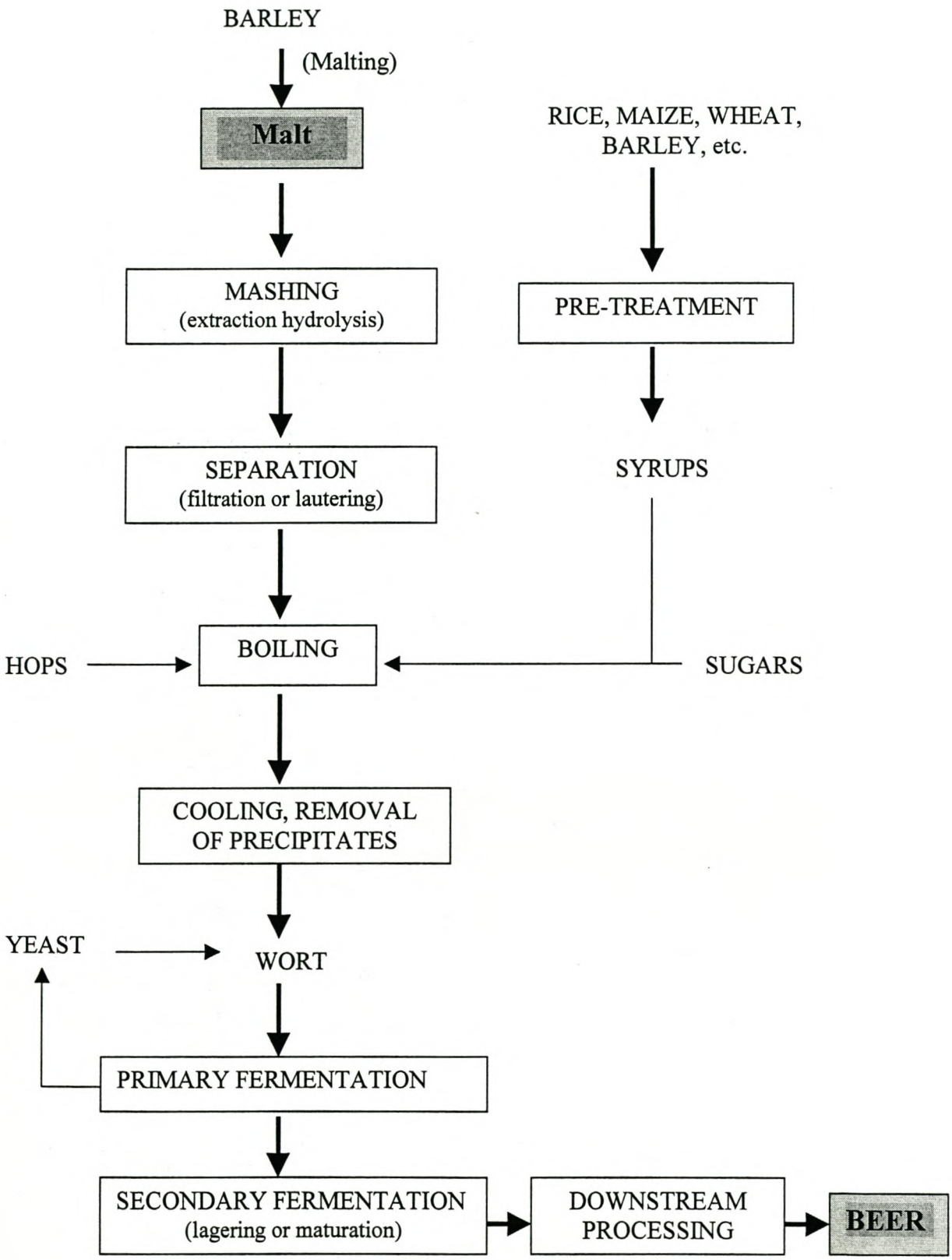

Fig. 6. Simplified scheme of the brewing process.

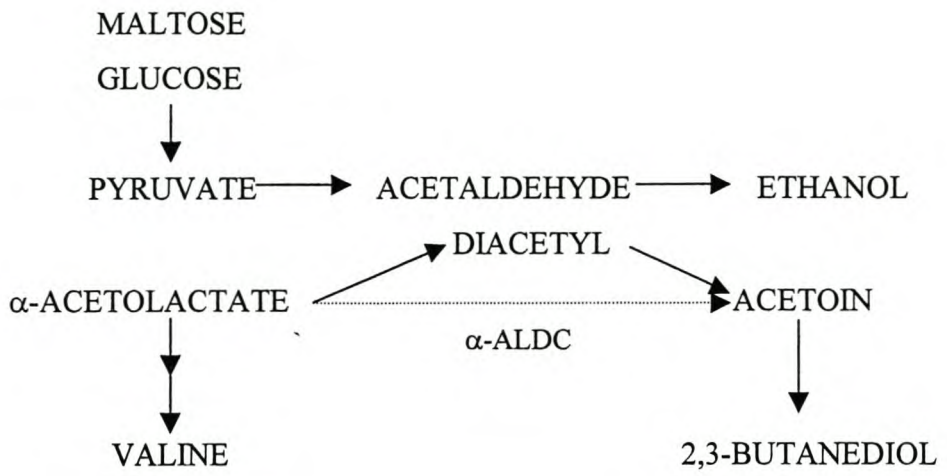

Fig. 7. Diacetyl is a key compound in maturation. Brewer's yeast lacks the enzyme α-acetolactate decarboxylase (α-ALDC). However, several bacteria produce α-ALDC and decarboxylises α-acetolactate directly to acetoin (dotted line).

CHANGES IN THE GRAIN DURING MALTING

As described previously, during the steeping phase the kernel takes up water. The main passage of water is at the micropyle near the embryo. This uptake of water causes swelling in the grain and leads to the growth of rootlets (Fig. 8b and c). The embryo increases in size and starts to respire more vigorously. With changes of the steep water the barley is cleansed and tannins are extracted from the husks.

On transition from steeping to germination the first signs of change is the emerging of main and secondary rootlets. The rootlets break through the embryo and grow up the dorsal side of the grain underneath the husk (pericarp). If sufficient time is available, the ascospire, the future stem of the barley plant, emerges at the apex of the grain (Gorg 1992; Eagles *et al.* 1995).

In the initial stages of germination the embryo needs nutrients and uses the starch inside the endosperm. The amount of existing enzymes increase and new enzymes are generated in the scutellum and aleurone layers. The enzymes modify the starchy endosperm, which becomes soft and rubs out. This activation and synthesis of enzymes is the most important process of malting.

An enzyme complex traditionally termed cytase, contributes to the second most important process, which consists of hydrolysing the hemicelluloses in the cell walls of the endosperm, thus leaving starchy contents of the cells available to microbial metabolism in the subsequent brewing process (Gorg 1992; Linko *et al.* 1998). Enzymes such as α-amylase, β-amylase, proteases and β-glucanase are also of importance (Gorg 1992; Noots *et al.* 1998). The amylase enzymes hydrolise the starch molecules into smaller fermentable sugars, while the proteases degrade proteins to easy metabolisable amino acids. The β-glucanase is responsible for hydrolysing β-glucane cell walls around the starch granules to render the starch available for degradation by amylases (Gorg 1992; Noots *et al.* 1998).

The moisture level of green malt is reduced from ca. 45% to 4% during kilning. The enzymes activated or synthesised in germination are to some extend destroyed. The enzyme content of the malt is one of the main factors, which determines the character of the malt. Aromatic and coloured compounds are formed, particularly if the temperature is increased when the moisture content of the green malt is still high. It is thus possible, by suitable choice of kilning temperatures and drying rates, to determine the character of the malt. Pale malt with a high enzyme content is used for brewing lagers, while dark malt with strong aroma but low enzyme content is used to brew ales and stouts (Linko *et al.* 1998).

The appearance of the kilned malt differs little from that of barley kernel (Fig. 8a and d), but the barley kernel is much harder than the brittle, easy to crush malt. This is due to the breakdown of the cell walls and protein matrix of the endosperm. The malt grain is lighter than the barley grain and is relatively prone to damage during transfer. The loss in weight (6 – 7%) is due to the loss in dry matter after the formation of rootlets and acrospires, which are removed after kilning, and due to respiration of the barley grain.

Fig. 8. Changes in the morphology of the kernel during the malting process: (a) Barley before malting; (b) plump or swollen barley after steeping (c) barley growing roots during germination (d) malted barley (malt). Photographed at SAM, Caledon.

MICROFLORA IN THE PRODUCTION CYCLE

Barley in the Field

The "field microflora" are those microorganisms found on barley prior to harvest and are mainly the microflora present in soil, vegetation and air (Riis *et al.* 1995). These microorganisms are mainly saprophytic and parasitic, can produce mycotoxins and can cause problems leading to blights or blemishes of barley. Gram-negative bacteria are the most abundant of the field microflora. The most common and abundant bacterial species always present are *Erwinia herbicola* (Bellimov *et al.* 1998). Yeast is usually the next most abundant, although the filamentous fungi may be dominant during the ripening stages (Bellimov *et al.* 1998). Different fungi usually occur throughout the different stages of growth of the barley plant. Genera such as *Alternaria*, *Aerobasidium*, *Fusarium* and *Cladosporium* are a few examples. *Aspergillus* and *Penicillium* spp. also occur, but they are mainly associated with stored barley (Haikara *et al.* 1977; Vaag 1991; Bellimov *et al.* 1998).

Contamination of barley by microorganisms originating from soil, vegetation, air, rain, insects and bird droppings occurs the moment the grain begins to germinate and continues throughout the growth period. Microorganisms trapped between the bactereols and caryopsis of the grain apparently have more favourable growth conditions than those on the outer surface of the grain (Jenkyn *et al.* 1992; Bellimov *et al.* 1998). Bacterial contamination is mainly through the faeces of birds and rats in the barley fields. The total counts of bacteria and moulds that occur in the barley seem to be lower in 'winter' barley than in 'spring' barley (Jenkyn *et al.* 1992). The type and abundance of the microflora present are to a large extend determined by the climatic conditions. The degree of microbial contamination is also determined by the cultivation practices, such as the use of crop protective agents like fungicides and pesticides, the percentage of acreage planted, and nitrogen fertilisers (Jenkyn *et al.* 1992; Noots *et al.* 1998).

Harvesting and Storage Microflora

In comparison with hand-trushing, combine harvesting leads to higher levels of contamination of the barley kernels, especially by the xerophylic *Penicillium* spp. (Linko *et al.* 1998; Noots *et al.*

1998). Possible sources of this contamination are through dust generated from the combine harvester. The types and numbers of contaminating microorganisms also depend upon the weather conditions at harvesting time. Heavy rainfall immediately before harvest disrupts the kernels at the furrows and leads to the development of fungi (Jenkyn *et al.* 1992).

The "seed microflora" is the most numerous and vigorous immediately after harvest. Sometimes the harvest is delayed to reduce moisture levels to limit mechanical damage to the grain and to keep drying costs low (Jenkyn *et al.* 1992). This delay results in higher microbial numbers, partly because the delay in drying allows for mold sporulation on the kernels, which in turn results in secondary cross contamination.

The composition of the microbial population of barley changes during storage, depending on storage time and environmental conditions. The development of the microflora is influenced by many factors, such as the moisture content and temperature of the grain mass, aeration, presence of chaff, and other materials such as broken grains and weed seeds and the occurrence of insects and mites in the grain bed (Jenkyn *et al.* 1992).

Under appropriate storage conditions, bacteria, yeast and fungi are not active due to the low moisture content of the barley. However, of all fungi, *Alternaria* spp. survive and grow the best under these conditions, while the endospores of *Bacillus* spp. are able to survive the storage conditions (Vaag 1991; Riis *et al.* 1995; Noots *et al.* 1998).

Malt Production

Natural contamination of barley kernels at the start of the malting process is a result of the field conditions under which the crop is grown and the post harvest history of the grain, as explained before. Several authors have described the microbiology of malting (Douglas and Flannigan 1988; Peters *et al.* 1988; Noots *et al.* 1998). These studies showed that unprocessed barley contains a significant microbial load consisting of a number of bacteria, yeast and fungi. The microbial load of the barley increases significantly during steeping and remains high during germination.

The activity and evolution of the microbial population during the different stages of the malting process depends mainly on the initial barley contamination before processing. Interactions between the members of the population, varying substrate characteristics, e.g. moisture content, availability of nutrients and favourable processing conditions, such as temperature and aeration, also play an important role. Further contamination may also occur due to specific microflora that may develop in the malting plant.

Steeping is the critical stage where microorganisms start to proliferate. The rapid hydration of the barley grain in conjunction with the leakage of nutrients into the steep liquor causes a rapid proliferation of bacteria, yeast and fungi. Proliferation of the microorganisms leads to the growth of molds and the activation of dormant spores which infects the grain bed (Follstad and Christensen 1962; Douglas and Flanigan 1988; Kelly and Briggs 1992). This occurs throughout steeping and germination and is encouraged by steep aeration. The microbial proliferation leads to a dense cover of various types of microorganisms, in particular in the case of damaged kernels. Changing the steep water removes a portion of the microbes and dissolved materials from the grain (Linko *et al.* 1998).

The progressive increase in the microbial population from dry barley to green malt is also attributed to the release of compounds which may be metabolised as a result of enzymatic activity in the germinating barley kernels (Follstad and Christensen 1962; Douglas and Flanigan 1988; Kelly and Briggs 1992). The viable count of bacteria and yeast reaches a maximum during germination. The temperature and moisture levels in the germination vessels favours growth and activity of fungi, especially during the second and third days (Warnock, 1971; Ziola *et al.* 1992).

The high temperature during kilning strongly influences the microbial load of the malt. Many microcolonies of bacteria and fungi were detected on the surface and husk of malt kernels (Peters *et al.* 1988). *Aspergillus* and *Pencillium* spp. are the dominant filamentous fungi on malt (Warnock, 1971; Ziola *et al.* 1992), although *Mucor* and *Rhizopus* spp. were also isolated (Ziola *et al.* 1992; Noots *et al.* 1998). During kilning, stress and nutrient limitation modifies microbial metabolism. Some lactobacilli may, for instance, oxidize lactate, which formed earlier during steeping and germination, to yield formic, acetic and succinic acid during glucose limitation (Peters *et al.* 1988). A sufficient reduction in lactic acid was measured after kilning, but the

levels of other acids showed little to no change (Peters *et al.* 1988; Noots *et al.* 1998). It seems likely that the volatile acids formed from lactic acid are removed during kilning so they could not influence the final malt pH (Noots *et al.* 1998)

The type of malting equipment used may also influence the evolution of the barley microflora. Saladin boxes favour the growth of bacteria and yeast, while floor malting favours the development of bacteria and molds (Douglas and Flanigan 1988; Noots *et al.* 1998). The microflora occurring on the malting equipment also contributes to the variety of microorganisms in the proliferating grain bed during malting. Lactic acid bacteria, for example, are some of the contaminants growing on the walls inside the steep and germination vessels. The yeast *Geotrichum candidum* normally occurs inside conical or flat bottom steep vessels and in Saladin germination boxes (Noots *et al.* 1998).

Apart from all the microorganisms mentioned in this section, a number of other bacteria and fungi have been isolated from barley and malt (Noots *et al.* 1998). Table 1 lists the most commonly found bacteria and fungi in barley, during the malting process, and on malt.

Table 1 Microorganisms detected on barley (B), barley malt intermediate (I), or malt (M)

	B	I	M
Bacteria[a]			
Gram-negative			
Alcaligenes spp.	-	+	-
Calvibacterium iranicum	+	+	+
Enterobacter agglomerans	+	-	+
Erwinia herbicola	+	+	+
Escherichia coli	+	+	+
Flavobacterium spp.	+	-	+
Pseudomonas fluorescens	+	+	+
Xanthomonas campestris	+	-	-
Gram-positive			
Actinomycetes spp.	+	+	+
Arthrobacter globiformis	+	+	+
Bacillus cereus	+	-	+
Corynebacterium fasciens	+	-	-
Lactobacillus spp.[#]	+	+	+
Leuconostoc mesenteroides subsp. *mesenteroides*	+	-	+
Micrococcus spp.	+	+	+
Pediococcus pentosaceus	+	-	+
Streptomyces spp.	+	-	-
Thermoactinomyces vulgaris	+	-	-

Table 1 (continued)

	B	I	M
Fungi[b]			
Ascomycota			
Alternaria spp. [#]	+	+	+
Artrinium phaeospermum	+	-	-
Aspergillus spp. [#]	+	+	+
Eurotium amstelodami	+	+	+
Botrytis cinerea	+	-	+
Candida catenula	-	+	+
Chaetomium globosum	+	-	-
Cladosporium cladosporioides	+	-	-
Cochliobolus spp.	+	+	-
Curvularia spp. [#]	+	-	-
Debaromyces hansenii	-	+	-
Didymella exitalis	+	-	-
Drechslera spp. [#]	+	-	-
Euperinicillium spp.	+	-	-
Fusarium spp. [#]	+	+	+
Geotrichum candidum	+	+	+
Gonatobotrys simplex	+	-	-
Hansenula polymorpha	+	-	-
Hyphopichia burtonii	+	-	-
Hyprocrea pulvinata	+	-	-
Micrọchium bolleyi	+	-	-
Neocosmospora spp.	+	-	-
Nigrospora oryzae	+	-	-
Papularia arundinis	+	-	-
Penicillium spp. [#]	+	+	+
Phoma herbarum	+	-	+
Pyrenophora teres	+	+	+
Saccharomyces spp.	+	-	-
Scopulariopsis brevicaulis	+	-	-
Septoria nodorum	+	-	-
Sordaria fimicola	+	-	-
Stemphylium consortiale	+	-	-
Talaromyces emersonii	+	-	-
Thermoascus crustaceus	+	-	-
Thielavia sependonium	+	-	-
Torulopsis candida	+	-	-
Trichoderma viride	+	-	-
Willopsis californica	+	-	-
Basidiomycota			
Cryptococcus albidus	+	-	-
Rhizoctonia spp.	+	-	-
Zygomycota			
Absidia corymbifera	+	-	+
Absidia ramosa	+	-	-
Rhizopus spp.	+	-	+

Table 1 (continue)

	B	I	M
Fungi[b]			
Syncephalastrum spp.	+	-	-
Thamnidium elegans	+	-	-
Mitosporic fungi			
Acremoniella altra	+	-	-
Acremonium strictum	+	-	-
Arthrobotrys superba	+	-	-
Aureobasidium pullulans	+	+	-
Cephalosporium spp.	+	+	+
Doranomyces spp.	+	-	-
Epicoccum spp.	+	+	+
Gliocladium roseum	-	+	-
Harzia acremonidoides	+	-	-
Helminthosporium spp.	+	+	+
Hormodendrum spp.	+	-	-
Monilia spp.	+	-	-
Papulaspora spp.	+	-	-
Sclerotium spp.	+	-	-
Septonema spp.	+	-	-
Spicaria spp.	+	-	-
Sporobolomyces roseus	+	-	-
Thermonyces lanuginosus	+	-	-
Thielaviopsis spp.	+	-	-
Torula herbarum	+	-	-
Trichosporon beigelii	+	+	+
Trichothecium roseum	+	+	+
Ulocladium atrum	+	-	-
Verticillium lecanii	+	+	-
Wallemia sebi	+	-	-

* Adapted from Noots *et al.* (1998)
[#] More than 7 different species were detected.
[a] Exceptionally coliforms, *Escherichia coli* and fecal streptococci are found.

REFERENCES

Bellimov, A.A., Kunakova, A.M., Gruzdeva, E.V. (1998) Influence of soil pH on the interaction of associative bacteria with barley. *Microbiology* **67**, 561-568.

Biovin, P., Malanda, M. (1996) Microflora management during malting, The future of biotechnology in the barley- malt- beer chain. In *Technical Quarterly of the Masters Brewer's Association of the Americas*, 97-109.

Bol, J., Klopper, H.A., Vermiere, M.E., Motshagen, M.E. (1985) Relation between the microflora of barley and malt quality. *Proceedings of the European Brewery Convention Congress*, Helinski, 643-650.

Douglas, P.E., Flanigan, B. (1988) A microbial evolution of barley malt production. *Journal of the Institute of Brew*ing **94**, 85-88.

Eagles, H.A., Bedggood, A.G., Panozzo, J.F., Martin, P.J. (1995) Cultivar and environmental effects on malting quality in barley. *Australian Journal of Agricultural Research* **46**, 831-844.

Follstad, M.N., Christensen, C.M. (1962) Microflora of barley kernels. *Applied Microbiology* **10**, 331-336.

Gorg, A., Weiss, W., Postel, W. (1992) Qualitative and quantitative changes in barley seed protein patterns during the malting process analyzed by sodium dodecyl sulfate- poli-acrylamide gel electrophoreses with respect to malting quality. *Electrophoresis* **13**, 787-797.

Haikara, A., Home, S. (1991) Mash filtration difficulties caused by split barley kernels: A microbiological problem. *Proceedings of the European Brewery Convention Congress*, Lisbon, 537-546.

Haikara, A., Makinen, V., Hakulinen, R. (1977) On the microflora of barley after harvesting, during storage and in malting. *Proceedings of the European Brewery Convention Congress,* Amsterdam, 35-47.

Hough, J., Kleyn, J. (1971) The microbiology of brewing. *Annual Reviews in Microbiology* **25**, 583- 608.

Jenkyn, J.F., Carter, N., Darby, R.J., Gutteridge, R.J., Mullen, L.A., Plumb, R.T., Ross, G.J.S., Todd, F.W., Widdowson, F.V., Wood, D.W. (1992) Effect of seven factors on the growth and yield of winter barley grown as a third consecutive take-all susceptible crop and of growing the barley oats or a fallow. *Journal of Agricultural Science* **119**, 303-311.

Kelly, L., Briggs, D.E. (1992) The influence of grain microflora on the germinative physiology of barley, *Journal of the Institute of Brew*ing **98**, 395-400.

Linko, M., Haikara, A., Ritala, A., Penttila, M. (1998) Recent advantages in the malting and brewing industry. *Journal of Biotechnology,* **65**, 85-98.

Noots, I., Delcour, J.A., Michiels, W. (1998) From field barley to malt: detection and specification of microbial activity for quality aspects. *Critical Reviews in Microbiol*ogy **25**, 121-253.

Peters, H.I., Flannigan, B., Austin, B. (1988) Quantitative and qualitative studies of the microflora of barley malt production. *Journal of Applied Bacteriol*ogy **65**, 279-297.

Riis, P., Vraag, P., Peetz, J. (1995) Influence of barley variety on *Fusarium* field infection. *Proceedings of the European Brewery Convention Congress,* Brussels, 117-125.

Smith, A.R.W., Simpson, W.J. (1992) Factors affecting antibacterial activity of hop compounds and their derivatives, *Journal of Applied Bacteriology* **72**, 327-334.

Vaag, P. (1991) Immunological detection of *Fusarium* in barley and malt. *Proceedings of the European Brewery Convention Congress,* Lisbon, 553-561.

Warnock, D.W. (1971) Assay of fungal mycelium in grains of barley, including the use of fluorescent antibody technique for individual fungal species. *Journal of General Microbiol*ogy **67**, 197-205.

Ziola, B., Whiting, M., Crichlow, M. and Ingledew, W.M. (1992) Detection of *Pediococcus* spp. in brewing yeast by rapid immunoassay. *Applied and Environmental Microbiology,* **58**, 713-716.

3. TAXONOMY OF LACTIC ACID BACTERIA WITH SPECIAL EMPHASIS ON SPECIES ISOLATED FROM MALT AND THE EFFECT THEY HAVE ON MALTING AND BREWING

TAXONOMY OF LACTIC ACID BACTERIA WITH SPECIAL EMPHASIS ON SPECIES ISOLATED FROM MALT AND THE EFFECT THEY HAVE ON MALTING AND BREWING

INTRODUCTION

The group lactic acid bacteria (LAB) is a member of the *Clostridium-Bacillus* subdivision of the Gram-positive Eubacteria (De Vuyst and Vandamme 1994, Holzapfel and Wood 1995). This subdivision includes the genera *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Streptococcus*, *Carnobacterium*, *Oenococcus*, *Vagococcus* and *Weissella* (De Vuyst and Vandamme 1994,Vandamme *et.al* 1996). These bacteria are Gram-positive, non spore-forming cocci, coccobacilli or rods. They are non-motile, prefer a non-aerobic habitat and do not reduce nitrate (Vandamme *et.al* 1996). Lactic acid bacteria are generally catalase-negative and usually lack cytochromes, although some species produce pseudo-catalase when grown in the presence of low sugar concentrations (De Vuyst and Vandamme 1994). A few strains produce catalase in media containing blood (Aguirre and Collins 1993).

Habitat

Lactic acid bacteria are widespread in nature and are found in habitats with high concentrations of soluble carbohydrates, protein breakdown products, vitamins and a low oxygen tension. The natural habitat of these bacteria is plants, as evident from the many species isolated from vegetables, fruit, silage, dough, wine, beer and other traditional fermented plant material (De Vuyst and Vandamme 1994). However, a number of species were also isolated from milk and dairy products, fermented meats, sewage and the intestinal, genital and respiratory tracts of man and animals (Allison and Galloway 1988; Aguirre and Collins 1993).

Commercial Significance

Lactic acid bacteria play an important role in the spoilage of processed and fermented foods, and beverages. Examples include souring and off-flavours in meat products and the spoilage of wine, beer, and fruit juices by *Pediococcus*, *Leuconostoc* and *Lactobacillus* spp. These organisms cause cloudiness and often produce off-flavours and polymers (Aguirre and Collins 1993).

Although lactic acid bacteria are generally regarded as safe (GRAS-status), some pathogenic species are found in the genus *Streptococcus* (Aguirre and Collins 1993). Several lactic acid

bacteria are responsible for the development of organoleptic compounds and may also contribute to the preservation of a wide range of food products (Aguirre and Collins 1993, De Vuyst and Vandamme 1994). These strains are particularly suitable as antagonistic microorganisms in foods, since they are capable of inhibiting other foodborne bacteria by a variety of means, including production of organic acids (e.g. lactic acid), hydrogen peroxide or bacteriocins, i.e. antimicrobial peptides or proteins (Aguirre and Collins 1993, De Vuyst and Vandamme 1994). LAB are also used as starter cultures in various food fermentations (Tables 1 and 2).

Table 1. Commercial significance of metabolic products of lactic acid bacteria (Holzapfel *et al.* 1995)

Metabolite	Beneficial	Deleterious
Lactic acid	Preservation Sensory improvement Enhancement of digestion and of nutrient uptake	Acidification
Acetic acid	Aroma	Off-taste
Diacetyl/acetoin	Aroma (dairy products)	Off-taste (beer)
CO_2	Preservation	Discolouration Greening
Biogenic amines	-	Health (allergies)
Slime	Stabilization (e.g. yogurt)	Sensory
Methanethiol and H_2S	Aroma	Sensory (off-taste and odor)
Bacteriocins	Preservation (inhibition of closely related bacteria)	Inhibition of beneficial lactic acid bacteria
Wide-spectrum antimicrobials	Inhibition of pathogens and spoilage microorganisms	Resistance of intestinal microorganisms

Table 2. Lactic acid bacteria used in food fermentations (Aguirre and Collins 1993)

Food/product	Raw ingredients	Microorganisms
Dairy products:		
Acidophilus milk	Milk	*Lactobacillus acidophilus*
Bulgarian buttermilk	Milk	*Lactobacillus bulgaricus*
Ripened cheeses	Milk curd	lactobacilli, lactococci
Kefir	Milk	*Lactobacillus lactis, Lactobacillus bulgaricus*
Kummis	Mare's milk	*Lactobacillus bulgaricus, Lactobacillus leichmanii*
Taette	Milk	*Lactococcus lactis* subsp. *lactis*
Yogurt	Milk	*Lactobacillus bulgaricus*
Meat and fish products:		
Dry sausages	Pork, beef	Pediococci, *Lactobacillus plantarum, Lactobacillus brevis*
Semi-dry sausages	Beef	Pediococci
Burong dalag	Fish, rice	*Leuconostoc mesenteroides, Lactobacillus plantarum*
Izushi	Fish, rice, vegetables	Lactobacilli
Plant products:		
Kenkey	Corn	Lactobacilli
Ogi	Corn	*Lactobacillus plantarum, Lactococcus lactis* subsp. *lactis*
Olives	Green olives	*Lactobacillus plantarum*, pediococci, *Lactobacillus brevis, Leuconostoc mesenteroides*
Pickles	Cucumbers	Pediococci, *Lactobacillus plantarum*
Sauerkraut	Cabbage	*Lactobacillus plantarum, Leuconostoc mesenteroides*
Soy sauce	Soy beans	*Lactobacillus delbrueckii* subsp. *delbrueckii*
Wine	Grapes	*Oenococcus oeni*
Sake	Rice	*Lactobacillus sakei, Lactobacillus collinoides*
Breads:		
San Fransisco sourdough	Wheat flour	*Lactobacillus sanfransisco*
Sour pumpernickel	Wheat flour	*Leuconostoc mesenteroides*
Idli	Rice and bean flour	*Leuconostoc mesenteroides*

* Pot *et al.* (1994)

Metabolism

Lactic acid bacteria are nutritionally fastidious and as mentioned above, require carbohydrates, amino acids, peptides, nucleic acid derivatives and vitamins (Aguirre and Collins 1993). They are generally acid-tolerant or acidophilic (grow between pH 4.5 and pH 6.4). Many species have adapted the ability to grow under widely different environmental conditions (Aguirre and Collins 1993). They ferment carbohydrates to lactic acid as major end product. Several *Lactobacillus* spp. degrade organic acids, e.g. citric acid, tartaric acid and malic acid, via oxaloacetic acid and pyruvate to CO_2 and lactic acid (Kandler and Weiss 1986). Many species of the genera *Lactobacillus, Leuconostoc* and *Pediococcus* and *Oenococcus oeni* convert L-malic acid to L-lactic acid and CO_2 (Kandler and Weiss 1986).

Lactic acid bacteria are classified as homo- or heterofermentative according to their sugar (hexose or pentose) fermentation patterns. Homofermentative lactic acid bacteria ferment sugars (hexoses) via the Embden-Meyerhof (glycolysis) pathway. This fermentation results in lactate being the major end product - more than 85% (Kandler and Weiss 1986, Hammes *et al.* 1992). The heterofermentative lactic acid bacteria ferment sugars (hexoses or pentoses) by glycolysis or via the 6-phosphogluconate pathway. Hexoses are fermented to lactic acid, ethanol or acetic acid, while pentoses are fermented to lactic acid, ethanol or acetic acid and CO_2 (Kandler and Weiss 1986).

Homolactic LAB may become heterofermentative, depending on how pyruvate is utilised (Kandler and Weiss 1986). Pyruvate, which is intermediately formed by both homolactic and heterolactic pathways, may either be converted to acetic acid or to diacetyl and its derivatives, or with hexose limitation, homolactic fermentation may become a heterofermentation with acetic acid, ethanol and formic acid as main products (Kandler and Weiss 1986).

TAXONOMIC METHODS USED

Traditionally taxonomy of microorganisms was done by a variety of morphological and physiological methods. Currently taxonomy is primarily based on phenotypic (morphological and physiological) and genetic characteristics (Aguirre and Collins 1993, Klein *et al.* 1996).

Phenotypic methods

Morphology.

Morphological features of LAB include cellular and colony (form, colour and dimension) characteristics. Cell-wall composition (Scheifler and Kandler 1972), cellular fatty acids (Suzuki *et al.* 1993) and the structure of isoprenoid quinones (Collins and Jones 1981) are used to characterise Gram-negative and Gram-positive bacteria. Physiological features include the organisms' ability to grow at different temperatures, pH-levels, salt concentrations, growth in the presence of different chemicals (e.g. antimicrobial agents) and the metabolism of different compounds (Vandamme *et al.* 1996).

Fermentation.

The fermentative nature of LAB is of considerable interest, since this makes them excellent model systems for the study of energy transduction, solute transport and membrane biology (Axelsson 1993). An important characteristic used in the differentiation of LAB is the mode of glucose fermentation under specific conditions (Vandamme *et al.* 1996). Thus, for practical and taxonomic purposes LAB are divided into three metabolic categories: (i) Obligately homofermentative, (ii) facultative heterofermentative and (iii) obligately heterofermentative (Kandler and Weiss 1986). The grouping is according to their sugar (hexose and pentose) fermentation patterns and the main metabolic pathways they use (Table 3).

(i) Obligately homofermentative LAB (Group I). These bacteria lack the enzyme glucose 6-phosphate-dehydrogenase and 6-phosphogluconate-dehydrogenase and cannot utilize pentoses or gluconate (Kandler and Weiss 1986).

(ii) Facultatively heterofermentative LAB (Group II). These bacteria ferment hexoses almost exclusively to lactic acid by the Embden-Meyerhof pathway, or to lactic acid, acetic acid, ethanol, and formic acid under glucose limitation. Pentoses are fermented to lactic acid and acetic acid through an inducible pentose phosphoketolase (Kandler and Weiss 1986).

(iii) Obligately heterofermentative LAB (Group III). These bacteria lack the fructose diphosphate (FDP) -aldolase enzyme (Kandler and Weiss 1986). Pentoses are fermented to lactic acid and acetic acid. A pentose phosphoketolase is usually involved in both pathways.

The formation of the different isomeric forms of lactic acid during fermentation of glucose can be used to distinguish between leuconostocs and most heterofermentative lactobacilli, as the former produce only D(-) -lactic acid and the latter a racemate of D- and L-lactic acid (Axelsson 1993).

SDS-PAGE.

This technique groups bacteria by comparing their whole cell protein patterns obtained by highly standardised sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Apart from being a very useful technique in comparing a large number of strains, SDS-PAGE is extremely reliable (Pot *et al.* 1994, Vandamme *et al.* 1996). Large numbers of LAB can be compared and identified using this technique. Digitally processed electrophoretic patterns of representative strains can be stored on computer files to identify other unknown isolates (Descheemaeker *et al.* 1994). Comparison of the protein fingerprints gives a reliable measure of taxonomic relatedness. Disadvantages of this technique are that it is time consuming and the fact that extremely standardised and reproducible experimental conditions are required.

Genotypic Methods

Classification of LAB is becoming more dependent on sophisticated methods, especially genotypic methods to eliminate overlapping phenotypic characteristics among genera. Genotypic methods include DNA-base composition, DNA-homologies, 16S and 23S rRNA sequence analysis and RAPD-PCR (random amplified polimorphic DNA-polymerase chain reaction) (Collins *et al.* 1989; Pot *et al.* 1994).

In general, LAB has an average DNA-base composition of less than 50 mol% G+C (Vandamme *et al.* 1996). DNA-homology or DNA-DNA hybridization studies identify bacteria according to the sequence similarity between different genomes (Wayne *et al.* 1987). This technique is used to determine close relationships (at species and subspecies level) between different species and has been used in the description of new species (Wayne *et al.* 1987; Dicks *et al.* 1995; Van Reenen and Dicks 1996). The main disadvantage of this method is the large amount of genomic DNA needed. The technique is also labour intensive, time consuming and standardized methods do not always give the same result (Grimont *et al.* 1980; Collins *et al.* 1989).

Table 3. Subdivision of different LAB species, especially *Lactobacillus* spp., according to their phenotypic and phylogenetic assignments (Hammes and Vogel 1995)

Phylogenetic group	**Species in fermentation group**		
	Group I (obligately homofermentative)	**GroupII (facultatively heterofermentative)**	**Group III (obligately heterfermentative)**
		Lb. acetotolerans, Lact. hamsteri	
(*Lb. delbruekii* group)	*Lb. acidophilus, Lb. amylophilus, Lb. amylovorus, Lb. crispatus, Lb. delbruekii* subsp. *bulgaricus* ("*Lb. bulgaricus*"), subsp. *delbruekii*, subsp. *lactis* ("*Lb. lactis*"), *Lb. gallinarum, Lb. gasseri, Lb. helveticus* ("*Lact. jugurti*"), *Lb. jensenii, Lb. johnsonii, Lb. kefiranofaciens, Lb. kefirgranum*		
(*Lact. casei-Pediococcus* group)	*Lb. aviarius* subsp. *aviarius*, subsp. *araffinosus, Lb. farciminis, Lb. ruminis, Lb. mali* ("*Lb. yamanashiensis*"), *Lb. salivarius* subsp. *salicinus*, subsp. *salivarius, Lb. sharpae, P. damnosus, P. dextrinicum, P. parvulus*	*Lb. agilis, Lb. alimentarius, Lb. casei, Lb.. bifermentans, Lb. coryniformis*, subsp. *coryniformis*, subsp. *torquens, Lb. curvatus, Lb. graminis, Lb. homohiochii, Lb. intestinalis, Lb. murinus* ("*Lb. animalis*"), *Lb. paracasei* subsp. *paracasei*, subsp. *tolerans, Lb. pentosus, Lb. plantarum, Lb. rhamnosus, Lb. sakei* ("*Lb. bavaricus*"), *P. acidilactici, P. pentosaceus*	*Lb. brevis, Lb. buchneri, Lb. collinoides, Lb. fermentum* ("*Lb. cellobiosus*"), *Lb. fructivorans* ("*Lb. trichodes*"), *Lb. hilgardii* ("*Lb. vermiforme*"), *Lb. kefir, Lb. malefermentans, Lb. oris, Lb. parabuchneri, Lb. parakefir, Lb. pontis, Lb. reuteri, Lb. suebicus, Lb. sanfrancisco, Lb. vaccinostercus, Lb. vaginalis*
(*Leuconostoc* group)			*Lb. fructosus, Weissella confusus* ("*Lb. confusus*"), *W. halotolerans* ("*Lb. kandleri*"), *W. minor* ("*Lb. minor*"), *W. viridescens* ("*Lb. viridescens*"), *W. hellenica, W. paramesenteroides* ("*Le. paramesenteroides*"), *Le. amelibiosum, Le. argentinum, Le. lactis, Le. mesenteroides, Le. pseudomesenteroides, Le. gelidum, Le. carnosum, Le. fallax, O. oeni*
Other lactobacilli	*Lb. catenaformis, Lb. vitulinus, Lb. rogosae, Atopobium minutum* ("*Lb. minutus*"), *Atopobium rimae* ("*Lact. rimae*"), *Atopobium uli* ("*Lb. uli*"), *Carnobacterium divergens* ("*Lb. divergens*") ("*Lb. carnis*"), *C. piscicola* ("*Lb. piscicola*") ("*Lb. maltaromicus*"), *Lc. lactis* ("*Lb. hordniae*") ("*Lb. xylosus*")		

Lb. kefirgranum and *Lb. parakefir* have not been included in 16S rRNA sequence analysis. *Lb.* = *Lactobacillus* *P.* = *Pediococcus* *W.* = *Weissella* *Le.* = *Leuconostoc* *O.*= *Oenococcus* *Lc.* = *Lactococcus*

Ribosomal RNA (rRNA) analysis is more suitable for determining phylogenetic positions of species and genera (Collins *et al.* 1991; Collins and Wallbanks 1992). 16S rRNA contains highly conserved domains within a specific species (Woese 1987; Schleifer and Ludwig 1995b). It is also now possible to determine the sequence of long stretches of rRNA (~1500 bases of 16S rRNA) from bacteria (Aguirre and Collins 1993). Databases with published and some unpublished partial or complete sequences of 16S rRNA are available (Olsen *et al.* 1991; De Rijk *et al.* 1992). Comparisons of these sequences are currently the most powerful and accurate to determine the phylogenetic relationships among microorganisms (Collins *et al.* 1991; Collins and Wallbanks 1992; Aguirre and Collins 1993).

Results of rRNA sequencing or DNA-rRNA hybridizations led to the re-assignment of many LAB species to other taxa (Dicks *et al.* 1995; Vandamme *et al.* 1996). PCR and the use of appropriate primers for direct sequencing of nearly the entire 16S or 23S rDNA molecules are one of the most accurate methods used in bacterial phylogeny studies (Vandamme *et al.* 1996). The reliability of this technique depends on the size of the conserved elements, i.e. the larger the conserved elements, the more information they bear, and thus the more reliable the conclusions (Vandamme *et al.* 1996). Organisms that generally share more than 97% rRNA sequence similarity may belong to a single species (Stackebrandt *et al.* 1985; Stackebrandt and Ludwig 1994).

Advantages of ribosomal RNA sequencing are that rRNA molecules, in particular 16S and 23S rRNA, contain alternating sequences of more or less conserved regions. Probes can thus be designed for different levels of phylogenetic groups, i.e. from kingdom to species level (Schleifer and Ludwig 1995a and 1995b). Furthermore, these molecules are present in several copies (up to 10^4) in each cell (Stackebrandt and Leisack 1993).

The main disadvantage of ribosomal RNA sequencing is that cross-reactions may occur between closely related species because of the conserved nature of rRNA molecules. Variation in hybridisation procedures could create problems, especially when large numbers of strains are studied. Another disadvantage is that very complex patterns are often generated which are difficult to analyse and compare (Vandamme *et al.* 1996).

CONCLUSION

The classification of lactic acid bacteria into different genera is largely based on morphology, mode of glucose fermentation, growth at different temperatures, configuration of lactic acid produced, ability to grow at high salt concentrations, and acid or alkaline tolerance. Although classical phenotypic characterisation is still important, proper classification of LAB relying on molecular biology techniques for classification to genus and even to species level is still needed.

DNA-DNA hybridisation studies play an important role in determining inter- and intra-specific relationship among strains and have in some cases been the only way to resolve identification problems. Ribosomal RNA sequencing and hybridisation studies are nowadays used to reveal the phylogenetic relatedness among strains and species. SDS-PAGE of whole cell proteins proved to be reliable in elucidating relationships at species and subspecies level. The different taxonomic methods all have advantages and disadvantages. A good approach for taxonomic studies are thus to use combinations of all variable phenotypic and genetic techniques.

LACTIC ACID BACTERIA ISOLATED FROM MALT

Introduction

As mentioned earlier, the conditions during malting are favourable for the multiplication of microorganisms, especially lactic acid bacteria (LAB). Although literature on the effect these organisms have on the final product is scarce, their metabolic products influence malting and brewing quality (Haikara *et al.* 1977). LAB species associated with malting and brewing includes *Lactobacillus* spp., *Leuconostoc* spp., *Lactococcus* spp., *Pediococcus* spp. and *Wiessella* spp. (Hough and Kleyn 1971; O'Sullivan *et al.* 1998; Ziola *et al.* 1992). The latter are known to decrease the pH of steep water during malting and the pH of beer during brewing. Certain strains produce extracellular slime (called rope), which is a heteropolymer containing glucose, mannose, nucleic acid, and often protein (Hough and Kleyn 1971). Other undesirable effects include

turbidity and off-flavours in beer. Certain bacterial strains are even capable of flocculating yeast (Ziola *et al.* 1992).

1. The genus *Lactobacillus*

Lactobacilli are the most common spoilage organisms in unpasteurised beer and are Gram-positive, non-sporeforming, long slender rods or short bacilli. Gelatine is not liquefied, casein not digested and indole and H_2S are not produced (Kandler and Weiss, 1986). All species are catalase and cytochrome negative. Growth temperatures range between 2°C and 53°C, with the optimum between 30°C and 40°C (Kandler and Weiss, 1986). All species are strictly fermentative with complex nutritional requirements. Only lactobacilli detected in malting and brewing will be discussed in this section.

Lactobacillus spp.

Lactobacillus delbrueckii group

This group contains *Lb. delbrueckii* (the type species of the genus *Lactobacillus*), the seven species of the *Lactobacillus acidophilus* group (see below), *Lactobacillus acetotolerans*, *Lactobacillus hamsteri*, *Lactobacillus jensenii*, *Lactobacillus kefiranofaciens*, *Lactobacillus helveticus*, *Lactobacillus kefirgranum* and *Lactobacillus amylophilus*. All the species in this group are homofermentative. *Lb. delbrueckii* contains three subspecies, i.e. *L. delbrueckii* subsp. *bulgaricus, L. delbrueckii* subsp. *delbrueckii* and *L. delbrueckii* subsp. *lactis*, which cannot be discriminated by rRNA sequence analyses (Klein et al. 1998). Collins *et al.* (1991) has reported 16S rRNA homologies of 90.8 to 99.3% for this group. The DNA homology between these species are more than 80%.

Lactobacillus acidophilus group

The strains in this group are homofermentative and consist of 6 species, including *Lb. acidophilus, Lactobacillus crispatus*, *Lactobacillus amylovorus*, *Lactobacillus gallinarum, Lactobacillus gasseri* and *Lactobacillus johnsonii*. *Lb. acidophilus* was described as *Bacillus*

acidophilus and renamed by Hansen and Moquot (1970). *Lb. crispatus* was described in 1953 while *Lb. gasseri* and *Lb. johnsonii* were described between 1980 and 1992. Johnson *et al.* (1980) and Lauer *et al.* (1980) reported that these species cannot easily be differentiated by classical methods and used DNA-DNA homology to differentiate among their strains. DNA-DNA homology studies divided the strains in this group into two subgroups, as also confirmed by analysis of their total soluble cell protein patterns (Klein *et al.* 1998). These subgroups are *Lb. acidophilus sensu stricto* (group A1), *Lb. crispatus* (group A2), *Lb. amylovorus* (group A3) and *Lb. gallinarum* (group A4). The second subgroup contained *Lb. gasseri* (group B1) and *Lb. johnsonii* (group B2).

Lactobacillus casei group

The 37 species in this group are facultative heterofermentative and comprises *Lactobacillus zeae*, *Lactobacillus aviarius*, *Lactobacillus farciminis*, *Lactobacillus ruminis*, *Lactobacillus mali*, *Lactobacillus salivarius*, *Lactobacillus agilis*, *Lactobacillus alimentarius*, *Lactobacillus bifermentans*, *Lactobacillus coryniformis*, *Lactobacillus curvatus*, *Lactobacillus graminis*, *Lactobacillus homohiochii*, *Lactobacillus intestinalis*, *Lactobacillus murinus*, *Lactobacillus paracasei*, *Lactobacillus pentosus*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Lactobacillus sakei*, *Lactobacillus brevis*, *Lactobacillus buchneri*, *Lactobacillus collinoides*, *Lactobacillus fermentum*, *Lactobacillus fructivorans*, *Lactobacillus hilgardii*, *Lactobacillus kefir*, *Lactobacillus malefermentans*, *Lactobacillus oris*, *Lactobacillus parabuchneri*, *Lactobacillus parakefir*, *Lactobacillus pontis*, *Lactobacillus reuteri*, *Lactobacillus suebicus*, *Lactobacillus sanfrancisco*, *Lactobacillus vaccinostercus* and *Lactobacillus vaginalis* (Swings *et al.* 1996).

Historically this group comprised of only *Lb. casei*, which was divided into five subspecies, i.e. *Lb. casei* subsp. *casei*, *Lb. casei* subsp. *alactosus*, *Lb. casei* subsp. *pseudoplantarum*, *Lb. casei* subsp. *tolerans*, and *Lb. casei* subsp. *rhamnosus*. Collins *et al.* (1989) described the species *Lb. paracasei* and *Lb. rhamnosus*. *Lb. rhamnosus* consisted of the strains of the former *Lb. casei* subsp. *rhamnosus*, while *Lb. paracasei* comprised of *Lb. casei* subsp. *paracasei*, *Lb. casei* subsp. *alactosus*, *Lb. casei* subsp. *pseudoplantarum* and *Lb. casei* subsp. *tolerans*. However, the rejection of *L. paracasei* and its inclusion in the species *Lb. casei* has been proposed (Dicks *et al.* 1996). The latter authors also described a new species, *Lb. zeae* nom. rev. with the type strain being the

former type strain of *Lb. casei* (ATCC 393^T). The new type strain of *Lb. casei* was proposed as ATCC 334 (Dicks *et al.* 1996). Reclassification of these strains was based on high DNA homology values (80 to 100%) between the type strain of *Lb. casei (*ATCC 393^T) and *Lb. zeae*. The DNA homology between *Lb. casei* and *Lb. paracasei* is 40%, while *Lb. rhamnosus* shares a DNA homology between 30 and 50% with the latter two species (Collins *et al.* 1989).

Lactobacillus reuteri / fermentum group

A specific biotype of *Lb. fermentum* (biotype IIb) was first isolated in 1962, but were reclassified (Kandler *et al.* 1980) as *Lb. reuteri* according to DNA-DNA homology (Klein *et al.* 1998). *Lb. reuteri* and *Lb. fermentum* are phenotypically closely related and differentiation based on biochemical features is difficult. Differentiation between these two species is only possible through molecular methods.

Other lactobacilli

The obligately heterofermentative species include *Lactobacillus bifermentans*, *Lactobacillus buchneri*, *Lactobacillus collinoides*, *Lactobacillus fructoforans*, *Lactobacillus hilgardii*, *Lactobacillus kefir*, *Lactobacillus malefermentans*, *Lactobacillus oris*, *Lactobacillus panis*, *Lactobacillus parabuchneri*, *Lactobacillus pontis*, *Lactobacillus sanfrancisco*, *Lactobacillus suebicus*, *Lactobacillus vaccinostercus*, and *Lactobacillus vaginalis*.

2. The genus *Leuconostoc*

Leuconostocs are a diverse group of Gram-positive, catalase negative cocci, that share many characteristics with the genus *Lactobacillus* and other lactic acid bacteria. Apart from their typical irregular coccoid morphology, leuconostocs are distinguished from the heterofermentative lactobacilli primarilly by their inability to produce ammonia from argenine and the formation of only D(-)-lactate from glucose (Collins *et al.* 1993).

The genus *Leuconostoc* contains the following species: *Leuconostoc argentinum* (Dicks *et al.* 1993), *Leuconostoc mesenteroides* (with three subspecies *Le. mesenteroides* subsp. *mesenteroides, Le. mesenteroides* subsp. *dextranicum,* and *Le. mesenteroides* subsp. *cremoris*), *Leuconostoc lactis, Leuconostoc amelibiosum, Leuconostoc gelidum, Leuconostoc carnosum, Leuconostoc citreum, Leuconostoc pseudomesenteroides* and, *Oenococcus oeni* (previously *Leuconostoc oenos*). Numerical analysis of total soluble cell protein patterns, DNA-DNA hybridization studies and 16S rRNA sequencing studies are the only way to distinguish between the strains in this genus (Dicks *et al.* 1990).

3. The genus *Weissella*

Strains belonging to this genus are generally short Gram-positive, non-motile, catalase negative rods with rounded to tapered ends or coccoid cells occurring singly, in pairs or in short chains (Collins et al. 1993). The cells are acidoduric and can grow between 4^0C and 37^0C but not at 45^0C (except for some of the *Weissella confusa* strains). Growth occurs in 8% NaCl, but not in 10% NaCl. The genus is readily distinguished from the other homofermentative lactic acid bacteria by the formation of gas from carbohydrates.

Several *Leuconostoc* species, especially those in the *Le. paramesenteroides* group (which includes typical lactobacilli, i.e. *Lactobacillus confusus*, *Lactobacillus minor, Lactobacillus kandleri, Lactobacillus halotolerans* and *Lactobacillus viridescens*), have been reclassified as *Weissella* species. The genus consists of seven species, i.e. *Weissella confusa*, *Weissella halotolerans*, *Weissella kandleri*, *Weissella minor*, *Weissella paramesenteroides*, *Weissellla viridescens* and *Weissella helenica* (Collins *et al.* 1993, Kandler and Wiess 1986). The G+C content of the DNA of species within the genus is between 37 and 47mol% (Collins et al. 1993). The relatedness between the 23S rRNA gene sequences was one of the reasons the species was grouped into one genus (Collins et al. 1993).

4. The genus *Lactococcus*

Lactococci are Gram-positive non-motile cocci or ovoid cells that occur singly or in chains. Historically the lactococci belong to the lactic acid streptococci, but Schleifer *et al.* (1995a) described a new genus. The genus can be easily distinguished from the other Gram-positive cocci by the products produced from glucose fermentation (Schleifer *et al.* 1995b). The strains grow at temperatures between 5^0C and 37^0C with their optimum temperature being 30^0C. Lactococci are associated with food products and plant material and are not found in fecal material or soil. These bacteria are known for their ability to produce high concentrations of diacetyl. Their ability to produce diacetyl is their main form of beer spoilage.

The genus *Lactococcus* comprises the species *Lactococcus lactis* subsp. *lactis, Lactococcus lactis* subsp. *diacetylactis, Lactococcus lactis* subsp. *cremoris, Lactococcus lactis* subsp. *hordniae, Lactococcus garvieae, Lactococcus plantarum, Lactococcus rafinolactis* and *Lactococcus piscium* (Pot *et al.* 1994). *Lc. lactis* has been isolated from raw milk and milk products. *Lc. lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris* are the most important lactic acid bacteria used in the dairy industry (Stiles and Holtzapfel 1997). The latter two species are distinguished by growth in differential media and by numerical analysis of total soluble cell protein patterns (Pot *et al.* 1994). Acid production from carbohydrates and rRNA gene analysis can also be used to identify and differentiate between *Lactococcus* spp. (Pot *et al.* 1994).

5. The genus *Pediococcus*

Pediococci are small, spherical, Gram-positive bacteria which tend to grow in tetrads, although some cells could also occur single or in pairs. They are micro-aerophilic to anaerobic and like the other lactic acid bacteria, have fastidious nutrient requirements. These bacteria can not grow at pH levels lower than 4.1. Pediococci are heterofermentative and L-lactic acid is the only major product of metabolism. However, these bacteria, like the lactococci, are known to produce diacetyl as a secondary end product and this is there main potential of beer spoilage.

Pediococcus is a phylogenetically heterogenous genus comprising eight species (Collins and Jones 1981). *Pediococcus damnosus* and *Pediococcus inopinatus* are associated with beer and sauerkraut, while *Pediococcus parvulus, Pediococcus pentosaceus* and *Pediococcus acidilactici* are associated with vegetable material, milk and dairy products (Collins and Jones 1981). *Pediococcus halophilus* is closely related to the genus *Tetragenococcus* and plays an important role in soy sauce (Collins and Jones 1981). The species *Pediococcus urinae-equi* is more closely related to the genus *Aeromonas* (Collins and Jones 1981).

6. The genus *Enterococcus*

The genus *Enterococcus* became a separate genus 15 years ago, with *Streptococcus faecalis* and *Streptococcus faecium* renamed as *Enterococcus faecalis* and *Enterococcus faecium*, respectively (Devriese *et al.* 1995). Subsequently, chemotaxonomic and phylogenetic studies reclassified more *Streptococcus* spp. to the genus *Enterococcus*, *viz. Enterococcus avium, Enterococcus casseliflavus, Entercoccus durans, Enterococcus gallinarum, Enterococcus malodoratus, Enterococcus cecorum, Enterococcus saccharolyticus, Enterococcus columbae, Enterococcus dispar, Enterococcus flavescens, Enterococcus hirae, Enterococcus mundtii, Enterococcus pseudoavium, Enterococcus raffinosus* and *Enterococcus sulfurens*.

Enterococci are Gram-positive cocci that occur in pairs or in short chains. They are predominantly found in the intestines of humans and animals (Collins *et al.* 1989). These bacteria are chemoorganotrophic and have complex nutritional requirements. The main end product of fermentation is L(+)-lactic acid. These organisms grow between 10^0C and 45^0C, pH 9.6 and in the presence of 6.5% (m/v) NaCl, esculin is hydrolised and most strains are resistant to 40% (m/v) bile salts (Kandler and Weiss, 1986). Enterococci are used as indicators for feacal contamination of water and food and are believed to be pathogenic to humans and animals (Collins *et al.* 1989).

Within the genus *Enterococcus*, 16S rRNA sequence analysis has revealed the existence of several phylogenetically related groups (Devriese *et al.* 1995). The first group consists of *E. durans*, *E. faecium*, *E. hirae*, and *E. mundtii*. These species are distinguished from each other by

pigment production and biochemical characteristics. *Enterococcus* spp. are normally found in the gastro-intestinal tract (GIT) of humans and animals, frozen-, dried- and processed food, raw milk and plant material (Devriese *et al.* 1995).

The second subgroup consists of *E. avium, E. raffinosus, Enterococcus malodoratus* and *E. pseudoavium.* These species are distinguished from each other by carbohydrate fermentation and are mostly associated with animals and rarely isolated from humans (Devriese *et al.* 1995).

The third subgroup is *E. casselliflavus* and *E. gallinarum,* which is linked to the *E. avium* group. These organisms are motile and have a low resistance to the antibiotic vancomycin. They differ from each other by pigment formation, carbohydrate fermentation and haemolysis and occur mainly on plant material. *E. faecalis, E. saccharolyticus, E. sulfureus, E. cecorum* and *E. columbae* form separate branches of discent in the phylogenetic tree (Devriese *et al.* 1995). Biochemical characteristics, RNA-DNA hybridization and 16S rRNA sequencing are used to distinguish between the species in this genus (Devriese *et al.* 1995).

THE EFFECT OF LACTIC ACID BACTERIA ON MALTING AND BREWING

The proliferation of lactic acid bacteria (LAB) has long been accepted as an integral component of the malting process. However, little is published on the LAB of commercial malting and the specific effects these bacteria have on malting and brewing (Stars *et al.* 1993).

Previous studies indicated that the LAB could affect malt and beer quality and contribute to poor germination (Stars *et al.* 1993). High LAB populations decrease the germination rate, rootlet growth and α-amylase production in the germinated kernels (Kelly and Briggs 1992). The latter authors also suggested that even when mature barley is malted truly optimum results are not obtained because of the deleterious effects of the microflora present. The metabolic end products of LAB influence the barley and malt composition by both chemical and biochemical interactions. The effect these microorganisms has on malt presumably results from the interference with barley respiration and the secretion of enzymes during malting. Since the LAB population of barley

influences the final quality of malt, it may also influence the brewhouse performance of malt and the beer quality (Haikara and Home 1991).

Mash filtration problems

Intensive growth of LAB results in acidic off-flavour in mash and produce wort with low pH values. High LAB counts on malt derived from split barley kernels gave rise to difficulty in mash filtration and hazy wort (Haikara and Home 1991). Filtration problems are caused by the production of extracellular polysaccharides by *Le. mesenteroides* subsp. *mesenteroides*. Enterobacteria, pediococci and *Lactobacillus* spp. do not cause mash filtration problems.

Brewhouse Performance and Beer Quality

The industrial spoilage of beer and ales by microorganisms are commonly referred to as beer infections. These microorganisms cause product spoilage by producing metabolic end products that disturb the delicate balance between unused flavour-active wort components and metabolic products produced by *Saccaromyces cerevisiae* (Hough and Kleyn 1971; Jay 1992). *Lactobacillus* and *Pediococcus* spp. are the most common spoilage bacteria found in wort, beer, and pitching yeast. *Pediococcus* spp. are generally considered to be the most undesirable contaminant, with *P. damnosus* being the species responsible for 90% of all beer spoilage (Lawrence and Priest 1981; Jay 1992; Ziola *et al.* 1992). The *Lactobacillus* spp. commonly associated with beer spoilage include *Lb. brevis*, *Lb. delbrueckii*, *Lb. plantarum* and *Lb. leichmanii* (Ziola *et al.* 1992).The spoilage patterns of beer and ales may be classified into five groups: ropiness, sarcinae sickness, sourness, turbidity and super-attenuation.

Ropiness is the condition where the beer becomes characteristically viscous and pours with a "oily" stream. *L. brevis* and *P. damnosus* growing in the beer causes this problem by the production of extracellular gum or slime that renders the beer jelly-like (Jay 1992; Ziola *et al.* 1992).

Sarcinae sickness is caused by *P. damnosus*, *P. acidilactici* and certain *Lactococcus* spp. that produce a honey-like or buttery off-flavour. This characteristic odour is the result of the

production of diacetyl to concentrations higher than the taste threshold of 0.05 mg/l (Jay 1992; Ziola *et al.* 1992).

Sourness in beers is caused by the production of lactic acid and acetic acid by LAB growing in beer when the mash temperature decreases to temperatures below 50°C. These organisms are capable of oxidizing ethanol to acetic acid (Jay 1992; Ziola *et al.* 1992).

Turbidity and off-smell in beer are caused by several *Lactobacillus*, *Lactococcus* and *Pediococcus* spp. growing in the beer (Jay 1992; Ziola *et al.* 1992).

Super-attenuation is caused by *Lactobacillus brevis*, hydrolysing dextrins and starch in the beer to fermentable sugars. These sugars are fermented by the yeast and leads to a "thin" beer with a high CO_2 pressure after bottling (Jay 1992, Ziola *et al.* 1992).

Growth of LAB in beer is possible because of the favourable pH (between pH 4.5 and pH 6) and a good content of utilisable sugars. Some bacteria e.g. *Pediococcus* spp., of brewing origin are resistant to the bacteriostatic effect of hop resins and tolerate the pH and alcohol content in wort and beer. Pediococci are difficult to eradicate from contaminated brewery equipment, since commonly used sanitisers are not totally effective (Lawrence and Priest 1981; Ziola *et al.* 1992). Pediococci are slow growing in beer and generally manifest their spoilage capacity in beer only after long storage.

Lactic acid bacteria as starter cultures in malting

The use of lactic acid starter cultures in malting is based on the microbicidic compounds and enzymes produced by these organisms. Certain *Lb. plantarum* and *P. pentosaceus* strains are especially efficient for the restriction of harmful microorganisms when added to steeping waters of barley at a level of about 10^7 cells/g (Linko *et al.* 1998). The addition of *Lb. plantarum* as starter cultures in malting was shown to reduce the occurrence of *Fusarium* contamination. Lactic acid starter cultures also restrict the growth of harmful bacteria which compete with grain tissue for dissolved oxygen and may retard mash filtration (Haikara and Home 1991). The use of LAB

in malting has also led to significant improvements in the quality of malt. The addition of LAB into the steeping waters retarded the formation of deoxynivalenol (DON) during the malting process, thus limiting gushing of the beer produced by DON-infected malt. In the presence of *Lb. plantarum* and *Lb. acidophilus* starter cultures the DON content was decreased by 70% (Linko *et al.* 1998).

REFERENCES

Aguirre, M., Collins, M.D. (1993) Lactic acid bacteria and human clinical infection, *Journal of Applied Bacteriology* **75**, 95-107.

Allison, D., Galloway, A. (1988) Empynema of the gall bladder due to *Lactobacillus casei*. *Journal of Infection* **17**, 191-200.

Axellson, L.T. (1993) Lactic acid bacteria: classification and physiology. In *Handbook of New Bacterial Systematics* ed. Goodfellow, M. and O'Donnell, A.G. pp. 151-194. London: Academic Press Ltd.

Collins, M.D., Jones, D. (1981) Distribution of isoprenoid quinone structural types in bacteria and their taxonomic implications. *Microbiological Reviews* **45**, 316-354.

Collins, M.D., Phillips, B.A., Zanoni, P. (1989) Deoxyribonucleic acid homology studies of *Lactobacillus casei*, *Lactobacillus paracasei* sp. nov., subsp. *paracasei* and subsp. *tolerans* and *Lactobacillus rhamnosus* sp. nov. comb. nov. *International Journal of Systematic Bacteriology* **39**, 105-108.

Collins, M.D., Rodrigues, U.M., Ash, C., Aguirre, M., Farrow, J.A.E., Martinez-Murcia, A., Phillips, B.A., Williams, A.M., Wallbanks, S. (1991) Phylogenetic analysis of the genus *Lactobacillus* and related lactic acid bacteria as determined by reverse transcriptase sequencing of 16S rRNA. *FEMS Microbiology Letters* **77**, 5-12.

Collins, M.D., Wallbanks, S. (1992) Comparative sequence analysis of the 16S rRNA genes of *Lactobacillus minutus*, *Lactobacillus rimae*, and *Streptococcus parvulus*: proposal for the creation of a new genus *Atopobium*. *FEMS Microbiology Letters* **95**, 235-240.

Collins, M.D., Samelis, J., Metaxopoulos, J., Wallbanks, S. (1993) Taxonomic studies on some leuconostoc-like organisms from fermented sausages: description of a new genus *Weissella* from *Leuconostoc paramesenteroides* group of species. *Journal of Applied Bacteriology* **75**, 595-603.

De Rijk, P., Neefs, J.-M., Van de Peer, Y., De Wachter, R. (1992) Compilation of small ribosomal subunit RNA sequences. *Nucleic Acids Research* **20**, 2075-2089.

Descheemaeker, P., Pot, B., Ledeboer, M., Verrips, T., Kersters, K. (1994) Comparison of the *Lactococcus lactis* differential medium (DCL) and SDS-PAGE of whole-cell protein extracts for the identification of lactococci to subspecies level. *Systematic and Applied Microbiology* **17**, 459-466.

Devriese, L.A., Pot, B., Vandamme, L., Kerstens, K., Haesebrouck, F. (1995) Identification of *Enterococcus* species isolated from food products and animal origin. *International Journal of Food Microbiology* **26**, 187-197.

De Vuyst, L., Vandamme, E.J. (1994) Antimicrobial potential of lactic acid bacteria, pp. 91-142. In *Bacteriocins of Lactic Acid Bacteria, Microbiology, Genetics and Applications* ed. De Vuyst, L. Vandamme, E.J. pp.91-141. London: Blackie Academic and Professional.

Dicks, L.M.T., Fantuzzi, L., Gonzalez, F.C., Du Toit, M., Dellaglio, F. (1993). Leuconostoc argentinum sp. nov., isolated from Argentine raw milk. *International Journal of Systematic Microbiology* **43**, 347-351.

Dicks, L.M.T., van Vuuren, H.J.J, Dellaglio, F. (1990) Taxonomy of *Leuconostoc* species, particularly *Leuconostoc oenos*, as revealed by numerical analysis of total soluble cell protein

patterns, DNA base compositions, and DNA-DNA hybridizations. *International Journal of Systematic Bacteriology* **40**, 83-91.

Dicks, L.M.T., Dellaglio, F., Collins, M.D. (1995) Proposal to reclassify *Leuconostoc oenos* as *Oenococcus oeni* [corrig.] gen. nov., comb. nov. *International Journal of Systematic Bacteriology* **45**, 395-397.

Dicks, L.M.T., Du Plessis, E.M., Dellaglio, F., Lauer, E. (1996). Reclasification of *Lactobacillus casei* subsp. *casei* ATCC 393 and *Lactobacillus rhamnosus* ATCC 15820 as *Lactobacillus zeae* nom. rev., designation of ATCC 334 as the neotype of *L. casei* subsp. *.casei,* and rejection of the name *Lactobacillus paracasei. International Journal of Systematic Bacteriology* **46**, 337- 340.

Grimont, P.A.D., Popoff, M.Y., Grimont, F., Coynault, C., Lemelin, M. (1980) Reproducibility and correlation study of three deoxyribonucleic acid hybridization procedures. *Current Microbiology* **4**, 325-330.

Haikara, A., Home, S. (1991) Mash filtration difficulties caused by split barley kernels: a microbiological problem. *Proceedings of the European Brewery Convention Congress*, Lisbon, 537-546.

Haikara, A., Makinen, V., Hakulinen, R. (1977) On the microflora of barley after harvesting, during storage and in malting. *Proceedings of the European Brewery Convention Congress,* Amsterdam, 35-47.

Hammes, W.P., Weiss, N., Holzapfel, W.P. (1992) The genera *Lactobacillus* and *Carnobacterium*. In *The prokaryotes. A handbook on the biology of bacteria: ecophysiology, isolation, identification and applications* ed. Balows, A., Trüper, H.G., Dworkin, M., Harder, W. and Schleifer, K.H. pp. 1535-1594. New York: Springer-Verlag.

Hammes, W.P., Vogel, R.F. (1995) The genus *Lactobacillus*. In *The genera of lactic acid bacteria. The lactic acid bacteria Vol. 2*, ed. Wood, B.J.B. and Holzapfel, W.H. pp. 19-54. Glasgow: Blackie Academic & Professional

Hansen, P.A., Mocquot, G., (1970) Lactobacillus acidophilus (Moro) comb. nov. *International Journal of Systematic Bacteriology* **20**, 325-327.

Holzapfel, W.H., Wood, B.J.B. (1995) Lactic acid bacteria in contemporary perspective. In *The genera of lactic acid bacteria,* Vol. 2, ed. Wood, B.J.B. and Holzapfel, W.H. p. 8. Glasgow: Chapman and Hall.

Hough, J., Kleyn, J. (1971) The microbiology of brewing. *Annual Reviews in Microbiology*. **25**, 583- 608.

Jay, J.M. (1992) Spoilage of miscellaneous foods. In *Modern Food Microbiology* pp. 236-248. Chapman and Hall, 115 Fifth Avenue, New York, NY 10003.

Johnson, J.L., Phelps, C.F., Cummins, C.S., London, J., Gasser, F. (1980) Taxonomy of the *Lactobacillus acidophilus* group. *International Journal of Systematic Bacteriology* **30**, 53-68.

Kandler, O., Stetter, K.O., Kohl, R. (1980) *Lactobacillus reuterii* sp. nov., a new species of heterofermentative lactobacilli. *Zentralbl. Bakt. I Orig.* **C1**, 264-269.

Kandler, O., Weiss, N. (1986) Section 14, Regular, non-sporing Gram-positive rods. In *Bergey's manual of systematic bacteriology,* Vol. 2, ed. Sneath, P.H.A., Mair, N.S., Sharpe, M.E. and Holt, J.G. pp. 1208-1234. Baltimore: The Williams & Wilkins Co.

Kelly, L., Briggs, D.E. (1992) The influence of grain microflora on the germinative physiology of barley. *Journal of the Institute of Brew*ing **98**, 395-400.

Klein, G., Dicks, L.M.T., Pack, A., Hack, B., Zimmerman, K., Dellaglio, F., Reuter, G. (1996). Emended descriptions of *Lactobacillus sake* (Katagira, Kitahara, and Fukami) and *Lactobacillus curvatus* (Abo-Elnaga and Kandler): Numerical classification revealed by protein fingerprinting

and identification based on biochemical patterns and DNA-DNA hybridisations. *International Journal of Bacteriology* **46**, 367-376.

Klein, G., Pack, A., Bonaparte, C., Reuter, G. (1998) taxonomy and physiology of probiotic lactic acid bacteria. *International Journal of Food Microbiology* **41**, 103-125.

Lauer, E., Helming, C., Kandler, O. (1980) Heterogeniety of the species Lactobacillus accidophilus (Mono) Hansen and Moquoot as revealed by biochemiical characteristics and DNA-DNA hybridisations, *Zentralbl. Bakt. I Orig* **C1**, 150-168.

Lawrence, D.R., Priest, F.G. (1981) Identification of brewery cocci. *Proceedings of the European Brewery Convention Congress,* 217-227.

Linko, M., Haikara, A., Ritala, A., Penttila, M. (1998) Recent advantages in the malting and brewing industry. *Journal of Biotechnology,* **65**, 85-98.

Olsen, G.J., Larsen, G., Woese, C.R. (1991) The ribosomal RNA database project. *Nucleic Acids Research* **19**, 2017-2021.

O'Sullivan, T.F., Walsh, Y. O'Mahony, A., Fitzgerald, G.F., van Sinderen, D. (1998). A comparitive study of malthouse and brewhouse microflora. *Journal of the Institute of Brewing* **105**, 55-61.

Pot, B, Ludwig, W., Kersters, K., Schleifer, K.H. (1994) Taxonomy of lactic acid bacteria. Microbiology, genetics and applications. In *Bacteriocins of Lactic Acid Bacteria* ed. De Vuyst, L. and Vandamme, E.J. pp. 13-90. London: Chapman and Hall.

Schleifer, K.H., Kandler, O. (1972) Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriological Reviews* **36**, 407-477.

Schleifer, K.H., Ludwig, W. (1995a) Phylogenetic relationships of lactic acid bacteria. In *The genera of lactic acid bacteria. The lactic acid bacteria,* Vol. 2, ed. Wood, B.J.B. and Holzapfel, W.H. pp. 7-18. Glasgow: Blackie Academic & Professional Publishers.

Schleifer, K.H., Ludwig, W. (1995b) Phylogeny of the genus *Lactobacillus* and related genera. *Systematic and Applied Microbiology* **18**, 461-467.

Stackebrandt, E., Ludwig, E.W., Fox, G.E. (1985) 16S Ribosomal RNA oligonucleotide cataloging. *Methods in Microbiology* **18**, 75-107.

Stackebrandt, E., Ludwig, E.W. (1994) The importance of choosing out-group reference organisms in phylogenetic studies: the *Atopobium* case. *Systematic Applied Microbiology* **17**, 39-43.

Stackebrandt, E., Liesack, W. (1993) Nucleic acids and classification. In *Handbook of new bacterial systematics* ed. Goodfellow, M. and O'Donnell, A.G. pp. 151-194. London: Academic Press Ltd.

Stars, A.C., South, J.B., Smith, N.A. (1993) Influence of malting microflora on malt quality. *Proceedings of the European Brewery Convention Congress,* Oslo, 103-111.

Stiles, M.E., Holtzapfel, W.H., (1997) Review article. Lactic acid bacteria of foods and their current taxonomy. *International Journal of Food Microbiology* **36**, 1-29.

Swings, J., Vandamme, P., Pot, B., Gillis, M., de Vos, P., Kerstens, K. (1996) Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microbiological Reviews* **60**, 407-438.

Suzuki, K., Goodfellow, M., O'Donnell, A.G. (1993) Cell envelopes and classification. In *Handbook of new bacterial systematics* ed. Goodfellow, M. and O'Donnell, A.G. pp. 195-250. London: Academic Press Ltd.

Vandamme, P., Pot, B., Gillis, M., De Vos, P., Kersters, K., Swings, J. (1996) Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microbiological Reviews* **60**, 407-438.

Van Reenen, C.A., Dicks, L.M.T. (1996) Evaluation of numerical analysis of random amplified polymorphic DNA (RAPD)-PCR as a method to differentiate *Lactobacillus plantarum* and *Lactobacillus pentosus*. *Current Microbiology* **32**, 183-187.

Wayne, L.G., Brenner, D.J., Colwell, R.R., Grimont, P.A.D., Kandler, P., Krichevsky, M.I., Moore, L.H., Moore, W.E.C., Murray, R.G.E., Stackebrandt, E., Starr, M.P., Truper, H.G. (1987) Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *International Journal of Systematic Bacteriology* **37**, 463-464.

Woese, C.R. (1987) Bacterial evolution. *Microbiological Reviews* **51**, 221-271.

Ziola, B., Whiting, M., Crichlow, M. and Ingledew, W.M. (1992) Detection of *Pediococcus* spp. in brewing yeast by rapid immunoassay. *Applied and Environmental Microbiology,* **58**, 713-716.

ISOLATION, IDENTIFICATION AND CHANGES IN THE COMPOSITION OF LACTIC ACID BACTERIA DURING THE MALTING OF TWO DIFFERENT BARLEY CULTIVARS

Isolation, identification and changes in the composition of lactic acid bacteria during the malting of two different barley cultivars

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Abstract

Malt has a complex microbial population which changes as the malting process commences. Little is known about the proliferation of lactic acid bacteria (LAB) in each of the malting phases. In this study we determined the number of LAB present in the different phases of malting with Clipper and Prisma barley cultivars. The strains were identified to species level by using numerical analysis of total soluble cell protein patterns, RAPD-PCR banding patterns and 16S rRNA sequencing. The number of viable LAB in the barley before steep was higher in Prisma than Clipper (7.6×10^4 and 1.2×10^3 cfu/g, respectively). Despite this, the number of viable cells recorded in the first steep water was slightly higher for Clipper (9.0×10^5 cfu/g) than Prisma (5.5×10^5 cfu/g). More or less the same cell numbers were recorded for the two barley cultivars after the first and second dry stands. Both cultivars displayed more or less the same cell numbers (3.7×10^7 for Clipper and 3.2×10^7 cfu/g for Prisma) after the third day of germination. However, a higher number of LAB were detected in the kilned Prisma malt (6.9×10^4 cfu/g) than the Clipper malt (1.5×10^4 cfu/g). *Leuconostoc argentinum*, *Leuconostoc lactis* and *Weissella confusa* were the most predominant in both cultivars. A few strains were identified as *Weissella paramesenteroides* (four strains), *Lactobacillus casei* (five strains), *Lactococcus lactis* (five strains) and *Lactobacillus rhamnosus* (two strains). *Lb. casei* and *Lb. rhamnosus* were not isolated from the Prisma cultivar, whilst *W. paramesenteroides* and *Lc. lactis* were absent in the Clipper cultivar. Kilned malt of the Clipper cultivar contained predominantly *Le. argentinum*, whereas the Prisma cultivar contained mainly *Lc. Lactis*. To our knowledge, this is the first report of LAB in Clipper and Prisma barley and the various malting phases. The influence of the various groups of lactic acid bacteria on the fermenting ability of brewers' yeast is currently being determined.

Keywords: Malt; barley; lactic acid bacteria

1. Introduction

The malting process starts by soaking the barley in water to increase the moisture content to approx. 43-47% (m/v). This rapid hydration of the barley kernels and the secretion of nutrients from the kernels into the water, known as the steeping process, leads to the proliferation of various microorganisms, including lactic acid bacteria (Kelly and Briggs, 1992). Microbial growth continues throughout steeping and germination of the kernels and cell numbers increase faster during germination when enzymes convert residual carbohydrates to fermentable sugars (Petters et al., 1988). Most of the cells are killed during the drying (kilning) of the kernels.

The microbial population in barley is rather complex and consists of a number of different bacteria, yeast and filamentous fungi (Noots et al., 1999). However, little has been published on the bacterial population in the various phases of commercial malting. The numbers of bacteria on barley may increase 700-fold during the production of green malt (O'Sullivan et al., 1999). However, the proliferation of these bacteria are strongly influenced by the malting technique used, e.g. an increase in bacterial numbers was obtained with floor malting, compared to malting in saladin boxes (Douglas and Flannigan, 1988). Total bacterial counts between 1.4×10^6 and 7.0×10^6 cfu/g, and 1.5×10^6 and 1.9×10^6 cfu/g were reported in two separate studies (Douglas and Flannigan, 1988). Petters et al. (1988) reported an increase in *Lactobacillus* spp. from 10^2 cfu/kernel at the start of the malting process (steep) to 10^6 cfu/kernel during germination (i.e. green malt) and 10^5 cfu/kernel after kilning (i.e. screened malt).

In this paper we report on the isolation and identification of lactic acid bacteria (LAB) throughout the malting process of two barley cultivars, Clipper (local cultivar) and Prisma (imported cultivar). Both cultivars were malted at Southern Associated Maltsters (SAM), Caledon, South Africa. To our knowledge this is the first report on the species of LAB in Clipper and Prisma barley and the different malting phases.

2. Materials and methods

2.1. Isolation of lactic acid bacteria

Lactic acid bacteria were isolated from ten phases throughout the malting processes of two barley cultivars, Clipper and Prisma, malted at SAM, Caledon, South Africa. The samples were

taken from four individual runs of each cultivar to represent the following phases: dry barley before steep, water from the first steep water-stand, barley after draining of the first steep, water from the second steep water-stand, barley from the second steep water-stand, barley after draining of the second steep, barley from the first, second and third days of germination in the germination vessels (GV), and malt after kilning.

Barley and malt samples (approx. 1kg) were collected at seven points in the sampling vessels with a sterile cylindrical tube sampler. The steep-water samples (approx. 1L) were collected directly from the steep vessels using a sterile flask attached to a nylon string. The barley and malt samples (5g) were mashed in a warring blender (Warring Commercial), after which 1g was suspended in 9ml sterile distilled water and serially diluted. The steep water samples were also serially diluted in 9ml sterile distilled water.

The bacteria were isolated by spread-plating 100µl of each dilution onto MRS agar (Biolab, Biolab Diagnostics, Midrand, South Africa). Incubation was at 30°C for 24h. A duplicate set of plates were incubated in the presence of 20% CO_2, 10.9% H_2 and nitrogen as balance. Reference strains were obtained from the American Type Culture Collection (ATCC) and Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM). The reference strains were cultured as described in the respective culture collection catalogues.

2.2. Preliminary identification

Bacterial colonies were selected at random (approx. 5 colonies per plate) based on differences in morphology, i.e. size, form, elevation and border, and streaked out for pure cultures. Gram reaction, oxidase, indole and catalase activity tests were conducted on pure cultures as described by Dicks (1985). Gram-positive, catalase negative isolates were tested for the production of D(-) and L(+)-lactic acid by using the enzymatic kit of Boehringer Mannheim (Roche Molecular Chemicals GmbH, Mannheim, Germany). Carbon dioxide production from the fermentation of glucose and gluconate was determined according to the methods described by Dicks and Van Vuuren (1987). The isolates were classified into three groups, i.e. obligately homofermentative (no CO_2 from glucose or gluconate), facultatively heterofermentative (CO_2 from gluconate) and obligately heterofermentative (CO_2 from glucose and gluconate). Strains belonging to the genus *Lactobacillus* were distinguished from the genus *Leuconostoc* by the configuration of lactic acid, the production of NH_3 from arginine (Dicks, 1985), and carbohydrate

fermentation reactions as recorded by using the API 50 CHL system of bioMerieux (Marcy l'Etoile, France).

2.3. Numerical analysis of total soluble cell protein patterns

The isolates and reference strains were grown in MRS broth (Biolab) at 30°C for 24h. The methods described by Pot et al. (1994) were used to prepare whole cell protein extracts and separate the proteins on SDS-polyacrylamide gels. Normalization of the protein banding patterns and numerical analysis were performed as described by Pot et al. (1994), using the software package GelCompar version 4.0 (Applied Maths, Kortrijk, Belgium), as described by Vauterin and Vauterin (1992).

2.4. RAPD-PCR analysis

The DNA of selected strains was isolated according to the method of Dellaglio et al. (1973). Each PCR-reaction of 25µl contained 2.5µl (5mM) $MgCl_2$ (Roche Molecular Chemicals), 2.5µl 10x Reaction Buffer, 4µl of a (10mM/L) dNTP mixture (Roche Molecular Chemicals), 1µl (5U/µl) Taq-polymerase (Roche Molecular Chemicals), 5µl (1pM/µl) Primer, 2µl gDNA and 8µl sterile distilled H_2O. Three single 10 base primers [GGCATGACCT (OPL-01), TGGGCGTCAA (OPL-02) and CAGCACCCAC (RP-01)] were used. Amplification products were analysed by electrophoresis in 1.4% (m/v) agarose gels with TAE buffer (Sambrook et al., 1989). Lambda DNA, digested with *Eco*R1 and *Hin*dIII (Roche Molecular Chemicals), was used as molecular marker. Numerical analysis of the RAPD-PCR profiles was done according to the methods described by Du Plessis and Dicks (1995) and Van Reenen and Dicks (1996).

2.5. 16S rRNA sequencing

The method described by Collins et al. (1991) was used to perform 16S rRNA sequencing on representative strains selected from the protein profile clusters. PCR was used to amplify a 16S rRNA gene using conserved primers close to the 3' and 5' ends of this gene. The PCR products were purified by using a Prep-A-gene kit (Bio-Rad, Hercules, Ca., USA) according to the manufacturer's instructions and were sequenced by using a *Taq* Dye Deoxy terminator cycle

sequencing kit (Applied Biosystems, Inc. Foster City, USA) and a model 373A automatic sequencer (Applied Biosystems, Inc.). The closest known relatives of the new isolates were determined by performing sequence data base searches and the sequences of closely related strains were retrieved from GenBank or Ribosomal Database Project libraries. Sequences were aligned by using the program PILEUP (Devereux et al., 1984) and the alignment was corrected manually. Distance matrices were produced by using the programs PRETTY and DNADIST (using the Kimura-2 correction parameter) (Felsenstein, 1989). A phylogenetic tree was constructed according to the neighbour-joining method with the program NEIGHBOR (Felsenstein, 1989). The statistical significance of the groups obtained was assessed by bootstrapping (500 replicates) by using the programs DNABOOT, DNADIST, NEIGHBOR and CONSENSE (Felsenstein, 1989). The accession numbers of the nucleotide sequences used for 16S rRNA analyses are shown in Fig. 6.

3. Results and discussion

The cell counts of the LAB in the four individual runs of the two barley cultivars (Clipper and Prisma) did not vary by more than 5%. The mean value of the cell counts, obtained for the individual runs, was calculated for each phase studied (Table 1). In both cultivars the LAB counts steadily increased by one or two log cycles after the first contact with water and swelling of the kernels (from 1.2×10^3 to 9.0×10^5 for Clipper and from 7.6×10^4 to 5.5×10^5 for Prisma). Slightly less LAB were isolated from the barley in the first dry stand (4.3×10^5 for Clipper and 4.4×10^5 for Prisma), probably due to the lower water activity in the kernels. However, the cell numbers associated with the kernels seem to have recovered after the second steep to numbers as high as 2.4×10^7 and 8.4×10^7, as detected in samples taken from barley in the second dry stand. Similar results have been reported in other studies (Douglas and Flannigan, 1988; Petters et al., 1988; Noots et al., 1999). This stimulation of bacterial growth during germination is ascribed to the hydration of the barley kernel and the leakage of nutrients into the steep water, optimal growth temperatures in the grain bed due to grain respiration, and the availability of fermentable sugars due to optimal activity of the malt enzymes (Noots et al., 1999).

After kilning the LAB counts in both cultivars dropped significantly to almost the same numbers than observed in barley before malting. The decrease in cell numbers observed after the

third day of germination, from 3.7 x 10^7 to 1.5 x 10^4 for Clipper and from 3.2 x 10^7 to 6.9 x 10^4 for Prisma, is ascribed to the high temperatures during kilning (Noots et al., 1999).

A total of 67 Gram-positive, catalase negative bacteria were isolated throughout the malting process of both cultivars. Of these, 38 strains did not produce NH_3 from arginine, and produced mainly D(-)-lactic acid and CO_2 from glucose. Based on these results and their carbohydrate fermentation profiles, recorded from the API system (Table 2), all 38 strains were preliminary classified as members of the genus *Leuconostoc*. Seventeen strains produced NH_3 from arginine, CO_2 from glucose and D(-)- as well as L(+)-lactic acid. Although the strains were morphologically similar to *Leuconostoc* spp., their carbohydrate fermentation profiles were different (Table 2) and resembled that of Group III (obligately heterofermentative) *Lactobacillus* spp. (Kandler and Weiss, 1986). Seven strains differed from the other strains based on their sugar fermentation profiles (Table 2) and their inability to produce CO_2 from glucose. These strains were rod-shaped and were preliminary classified as homofermentative members of the genus *Lactobacillus*. Five strains were coccoid and their carbohydrate fermentation profiles (Table 2) closely resembled that recorded for lactic streptococci (Mundt, 1986), now *Lactococcus* spp.

Protein profile analysis grouped the 38 strains that were isolated from malt and preliminary classified as *Leuconostoc* spp. (Table 2) into two clusters (Fig. 1). Thirteen strains in cluster I grouped at $r \geq 0.87$ with the type strain of *Leuconostoc lactis* (DSM 20202^T) and *Le. lactis* DSM 20192. The remaining 25 strains clustered at $r \geq 0.86$ with the type strain of *Leuconostoc argentinum* (ATCC 51353^T) and two other strains of the same species (ATCC 51354 and ATCC 51355). Strains within the two clusters, representing the species *Le. lactis* and *Le. argentinum*, linked at $r = 0.78$ (Fig. 1), indicating that they are phenotypically related. Comparison of the whole cell protein patterns of only the type strains from the latter two species have shown an even closer phenotypic relationship at 87% (Björkroth et al., 2000). Results obtained by numerical analysis of RAPD-PCR profiles of representative strains selected from the two protein profile clusters in Fig. 1 clearly separated strains of *Le. lactis* from *Le. argentinum* (Fig. 2). This is contradictory to results obtained by ribotyping, which indicated that the two species are genetically closely related (Björkroth et al., 2000). However, sequencing of genes encoding 16S rRNA has separated strains of *Le. argentinum* from *Le. lactis* (Fig. 6). The *Leuconostoc* strains isolated in this study are thus regarded as being members of *Le. lactis* and *Le. argentinum* (Table 3).

Concluded from results obtained by numerical analysis of total soluble cell protein profiles, the 17 heterofermentative strains preliminary classified as *Lactobacillus* spp. (Table 2) are members of the genus *Weissella* (Fig. 3). Thirteen strains grouped with *Weissella confusa* ATCC 27646 and ATCC 10881 at a level of $r \geq 0.91$. The remaining four strains formed a tight grouping ($r \geq 0.96$) with *Weissella paramesenteroides* ATCC 33313 (cluster II, Fig. 3). Strains from the two clusters are related at $r = 0.90$. Since such high correlation values were recorded with the protein profiles, RAPD-PCR analysis were not performed. 16S rRNA sequencing (Fig. 6) confirmed the classification of the strains in clusters I and II as *W. confusa* and *W. paramesenteroides*, respectively (Table 3).

The seven homofermentative strains (no CO_2 production from glucose) isolated from malt (Table 2) were identified as *Lactobacillus rhamnosus* (two strains) and *Lactobacillus casei* (five strains) (clusters I and II, respectively, Fig. 4). Strains of the latter two species were not isolated from the Prisma cultivar. RAPD-PCR and 16S rRNA sequencing were not performed on the strains of *Lb. rhamnosus* and *Lb. casei*, since they grouped into two tight protein profile clusters ($r \geq 0.95$ and $r \geq 0.96$, respectively). Strains from the two clusters are phenotypically closely related ($r = 0.89$), confirming our previous findings (Dicks et al., 1996).

The five strains preliminary classified as members of the genus *Lactococcus* (Table 2) grouped in a tight cluster at $r \geq 0.95$ with *Lactococcus lactis* IL 1403 (Fig. 5) and were not subjected to RAPD-PCR and 16S rRNA sequencing.

Concluded from these results, the Clipper cultivar contained *Le. lactis, Le. argentinum, W. confusa, Lb. casei* and *Lb. rhamnosus*, but no *W. paramesenteroides* and *Lc. lactis*. The Prisma cultivar contained strains of *Le. lactis, Le. argentinum, W. confusa, W. paramesenteroides* and *Lc. lactis*, but no strains of *Lb. rhamnosus* and *Lb. casei*. The predominant bacterial species present in both barley cultivars before steeping were *Le. Lactis, Le. argentinum* and *W. confusa*. The same species were isolated from malt in the germination vessels, except that *Lb. rhamnosus* and *Lb. casei* were not present in the Prisma cultivar. Kilned malt of the Clipper cultivar contained predominantly *Le. argentinum,* whereas the Prisma cultivar contained mainly *Lc. Lactis*. It is not known if the species identified in this study have any influence on yeast fermentation, organoleptic properties of beer or contribute to spoilage. The influence of the various groups of lactic acid bacteria on the fermenting ability of brewers' yeast is currently being determined.

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References

Björkroth, K.J., Geisen, R., Schillinger, U., Weiss, N., de Vos, P., Holzapfel, W.H., Korkeala, H.J., Vandamme, P., 2000. Characterization of *Leuconostoc gasicomitatum* sp. nov., associated with spoiled raw tomato-marinated broiler meat strips packaged under modified-atmosphere conditions. Appl. Environ. Micobiol. 66, 3764-3772.

Collins, M.D., Rodrigues, U.M., Ash, C., Aguirre, M., Farrow, J.A.E., Martinez-Murcia, A., Phillips, B.A., Williams, A.M., Walbanks, S., 1991. Phylogenetic analysis of the genus *Lactobacillus* and related lactic acid bacteria as determined by reverse transcriptase sequencing of 16S rRNA. FEMS Microbiol. Lett. 77, 5-12.

Dellaglio, F., Bottazzi, V., Trovatelli, L.D., 1973. Deoxyribonucleic acid homology and base composition in some thermophilic lactobacilli. J. Gen. Microbiol. 74, 289-297.

Devereux, J., Haeberli, P., Smithies, O., 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res 12, 387-395.

Dicks, L.M.T., 1985. Taxonomy and physiology of heterofermentative *Lactobacillus* species including isolates from South African red wines. M.Sc. thesis, University of Stellenbosch, Stellenbosch, South Africa.

Dicks, L.M.T., Van Vuuren, H.J.J., 1987. A modification of the hot-tube method for the detection of carbon dioxide produced by heterofermentative *Lactobacillus* strains. J. Microbiol. Meth. 6, 273-275.

Dicks, L.M.T., Du Plessis, E.M., Dellaglio, F., Lauer, E., 1996. Reclasification of *Lactobacillus casei* subsp. *casei* ATCC 393 and *Lactobacillus rhamnosus* ATCC 15820 as *Lactobacillus zeae* nom. rev., designation of ATCC 334 as the neotype of *L. casei* subsp. *casei*, and rejection of the name *Lactobacillus paracasei*. Int. J. Syst. Bacteriol. 46, 337-340.

Du Plessis, E.M., Dicks, L.M.T., 1995. Evaluation of random amplified polymorphic DNA (RAPD)-PCR as a method to differentiate *Lactobacillus acidophilus*, *Lactobacillus*

crispatus, *Lactobacillus amylovorus, Lactobacillus gallinarum*, *Lactobacillus gasseri* and *Lactobacillus johnsonii*. Curr. Microbiol. 31, 144-118.

Douglas, P.E., Flannigan, B., 1988. A microbial evolution of barley malt production. J. Inst. Brew. 94, 85-88.

Felsenstein, J., 1989. PHYLIP-phylogeny inference package (version 3.2). Cladistics 5, 164-166.

Kandler, O., Weiss, N., 1986. Genus *Lactobacillus* Beijerinck 1901, 212AL. In: Sneath, P.H.A., Mair, N.S., Sharpe, M.E., Holt, J.G. (Eds.), Bergey's Manual of Systematic Bacteriology, vol. 2 ed. The Williams and Wilkins Co., Baltimore, pp.1209-1234.

Kelly, L., Briggs, D.E., 1992. The influence of grain microflora on the germinative physiology of barley. J. Inst. Brew. 98, 395-400.

Mundt, J.O., 1986. Lactic Acid Streptococci. In: Sneath, P.H.A., Mair, N.S., Sharpe, M.E., Holt, J.G. (Eds.), Bergey's Manual of Systematic Bacteriology, vol. 2 ed. The Williams and Wilkins Co., Baltimore, pp.1065-1068.

Noots, I., Delcour, J.A., Michiels, W., 1999. From field barley to malt: detection and specification of microbial activity for quality aspects. Crit. Rev. Microbiol. 25, 121-153.

O'Sullivan, T.F., Walsh, Y., O'Mahony, A., Fitzgerald, G.F., van Sinderen, D., 1999. A comparitive study of malthouse and brewhouse microflora. J. Inst. Brew. 105, 55-61.

Petters, H.I., Flannigan, B., Austen, B. 1988. Quantitative and qualitative studies of the microflora of barley malt production. J. Appl. Bacteriol. 65, 279-297.

Pot, B., Vandamme, P., Kersters, K., 1994. Analysis of electrophoretic whole organism protein fingerprints. In: Goodfellow, M., O' Donnell, A.G. (Eds.), Chemical Methods in Prokaryotic Systems, John Wiley and Sons Ltd., Chichester, pp. 493-521.

Sambrook, J, Fritsch, E.F., Maniatis, T. 1989. Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Van Reenen, C., Dicks, L.M.T., 1996. Evaluation of numerical analysis of random amplified polymorphic DNA (RAPD)-PCR as a method to differentiate *Lactobacillus plantarum* and *Lactobacillus pentosus*. Curr. Microbiol. 32, 183-187.

Vauterin, L., Vauterin, P., 1992. Computer-aided objective comparison of electrophoresis patterns for grouping and identification of microorganisms. Eur. Microbiol. 1, 37-41.

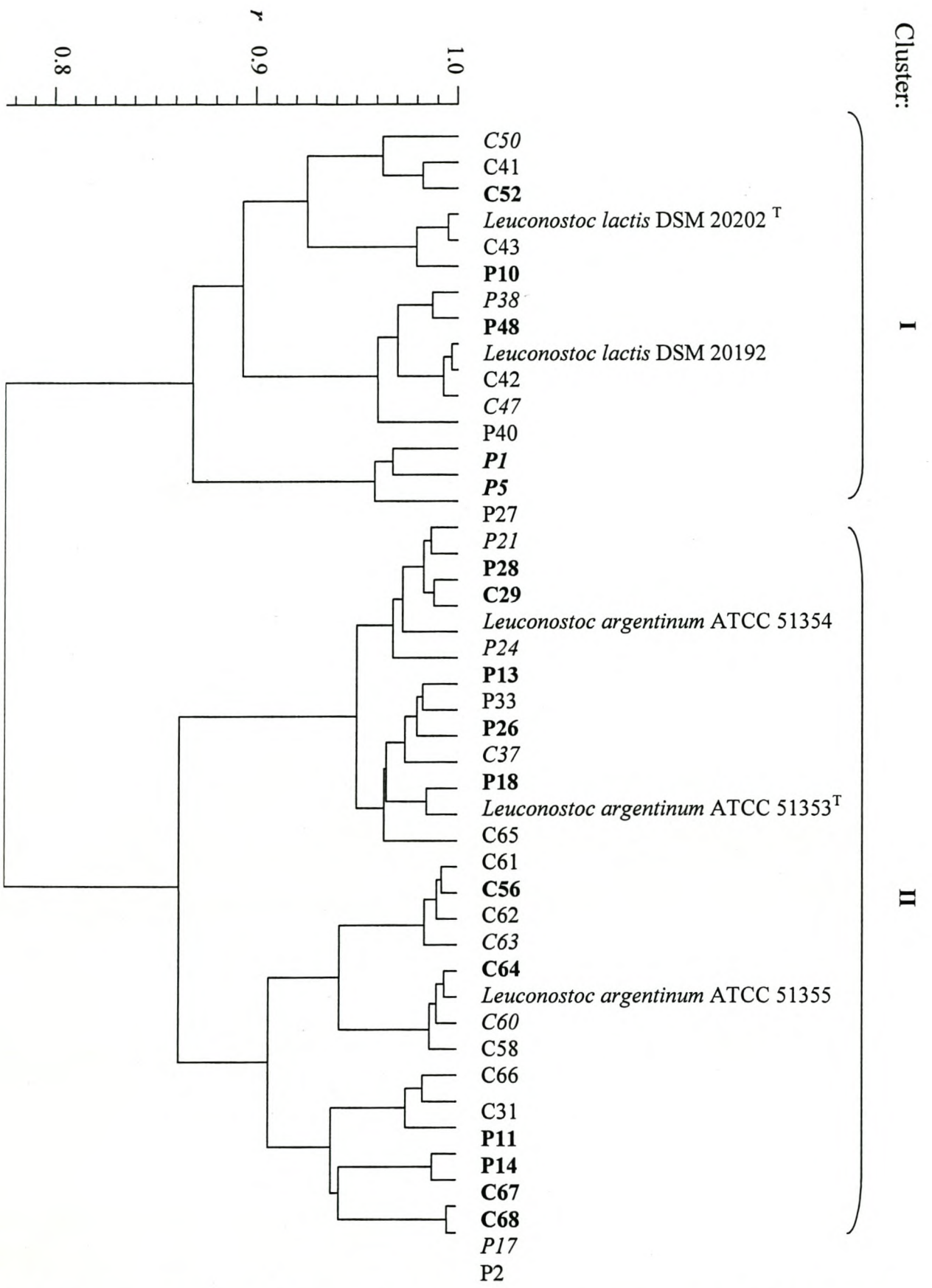

Fig. 1. Dendrogram showing the clustering of *Leuconostoc* spp. based on numerical analysis of total soluble cell protein patterns. Clustering was by UPGMA. Strains numbered with a "C" and "P" were isolated from Clipper and Prisma cultivars, respectively. Strain numbers in italics were subjected to RAPD-PCR and those in bold selected for 16S rRNA sequencing.

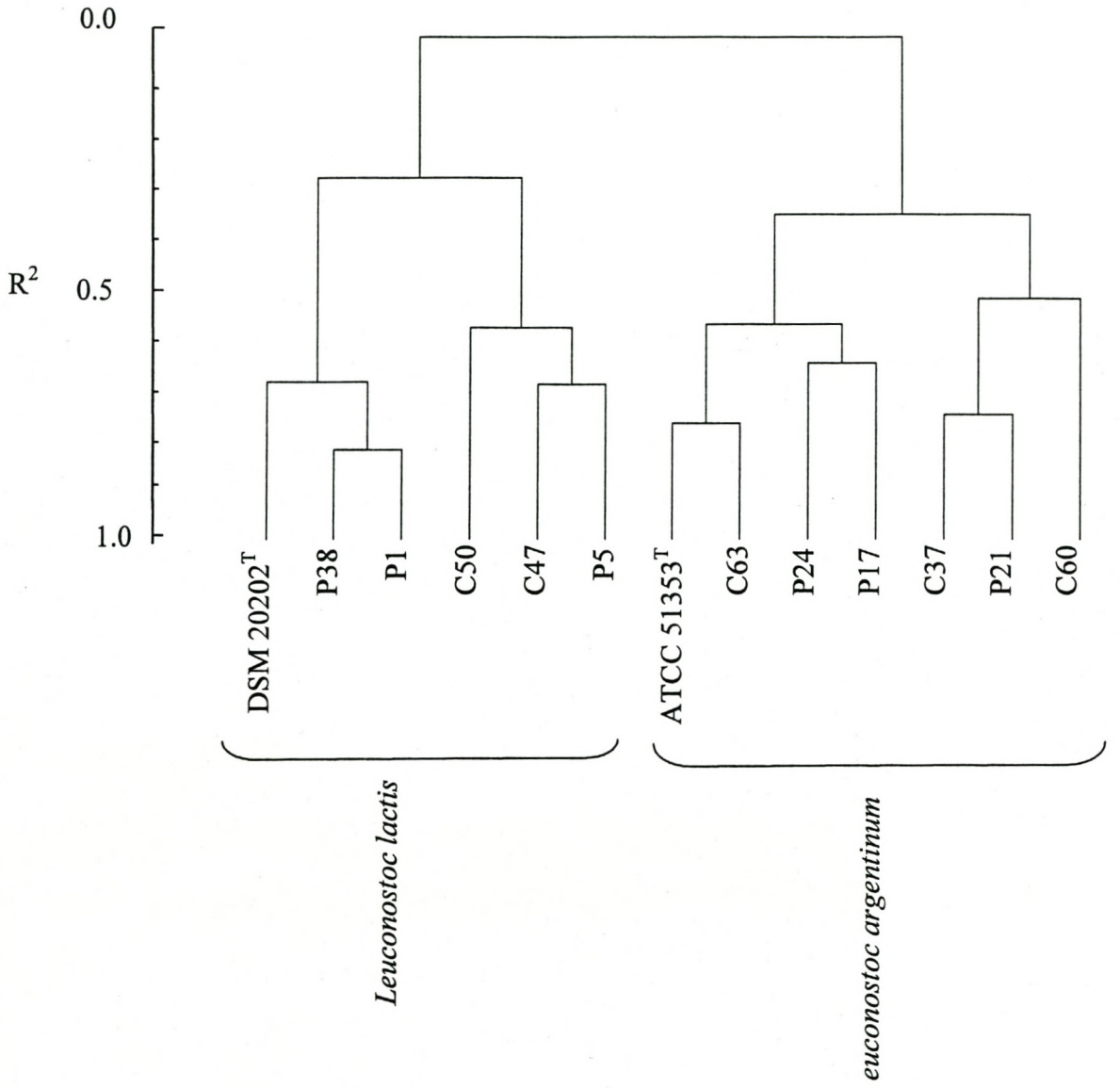

Fig. 2. Dendrogram showing the clustering of the *Leuconostoc* spp. obtained by numerical analysis of RAPD-PCR profiles. Strains were selected from Fig. 1. Clustering was by average linkage analysis.

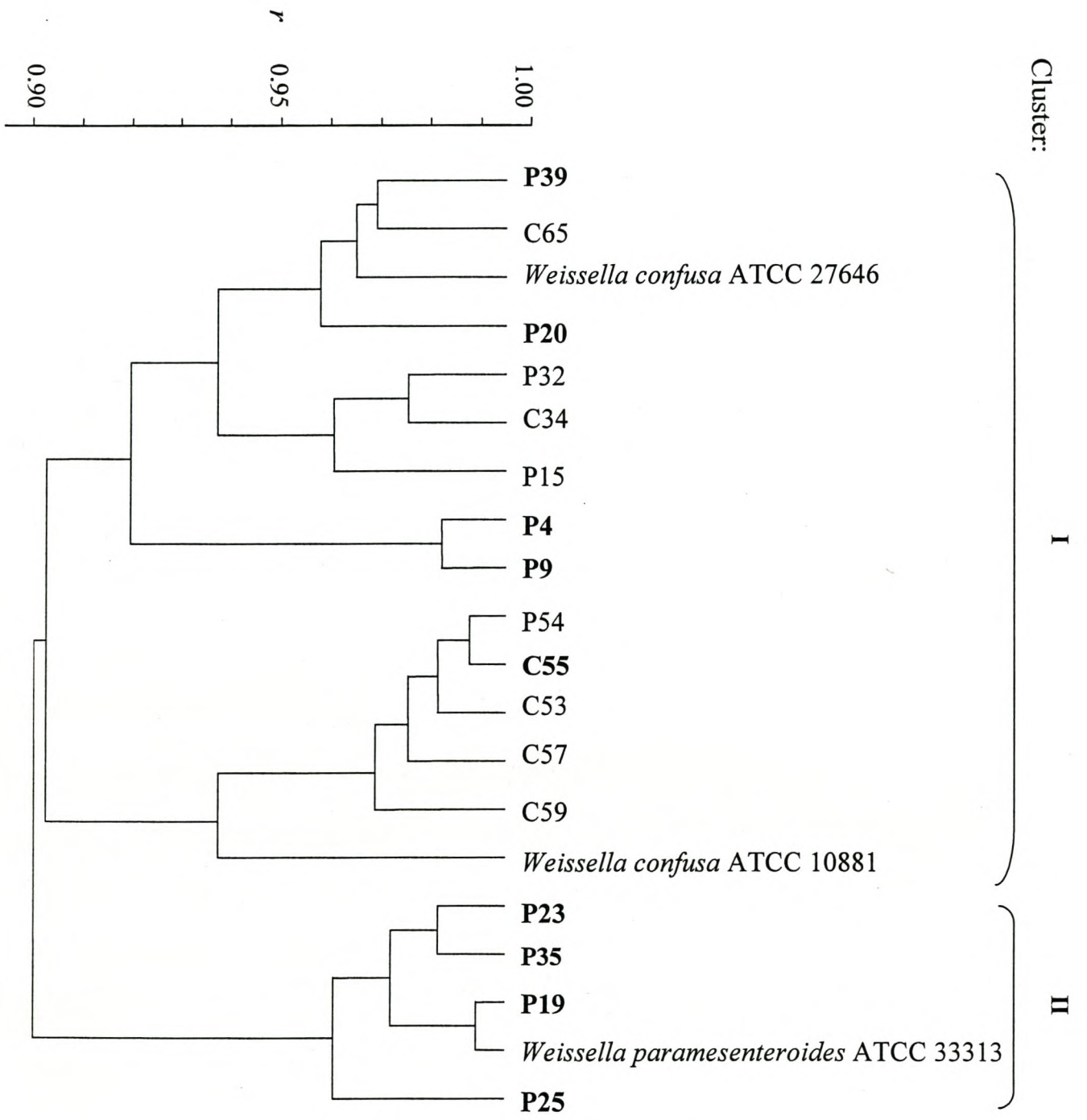

Fig. 3. Dendrogram showing the clustering of *Weissella* spp. based on numerical analysis of total soluble cell protein patterns. Clustering was by UPGMA. Strains numbered with a "C" and "P" were isolated from Clipper and Prisma cultivars, respectively. Strain numbers in bold selected for 16S rRNA sequencing.

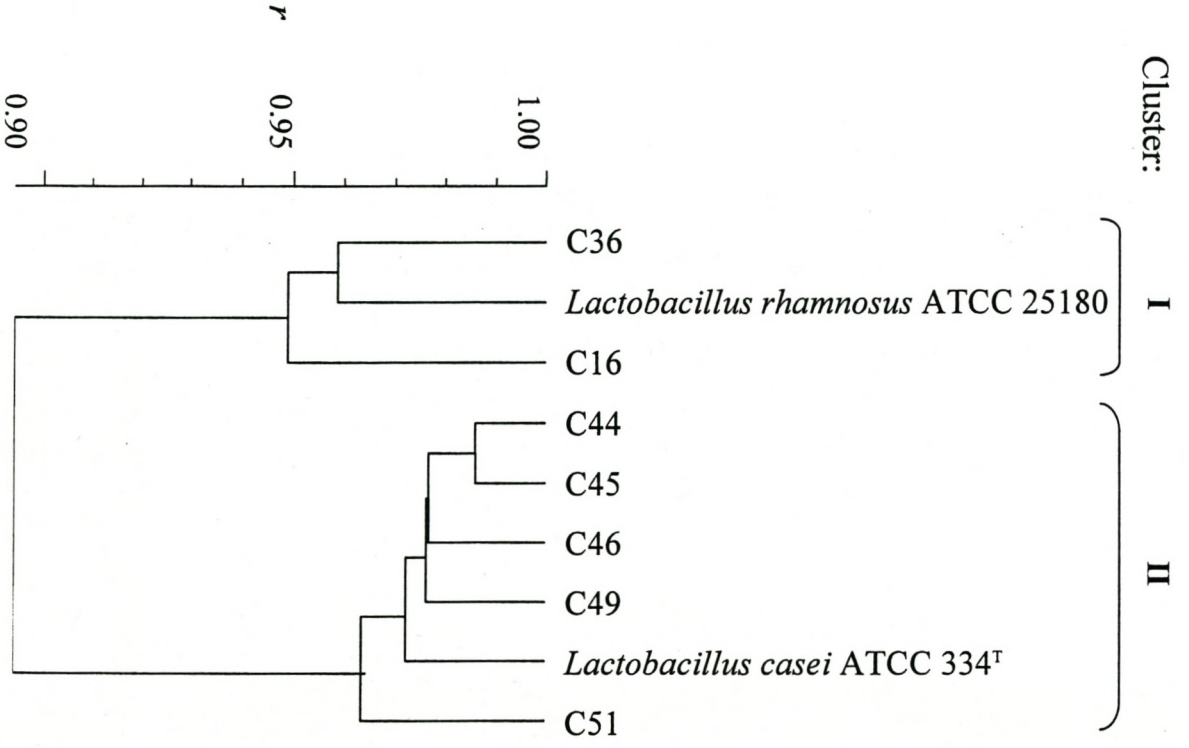

Fig. 4. Dendrogram showing the clustering of the homofermentative *Lactobacillus* spp. obtained by numerical analysis of total soluble cell protein patterns. All strains were isolated from the Clipper cultivar. Clustering was by UPGMA.

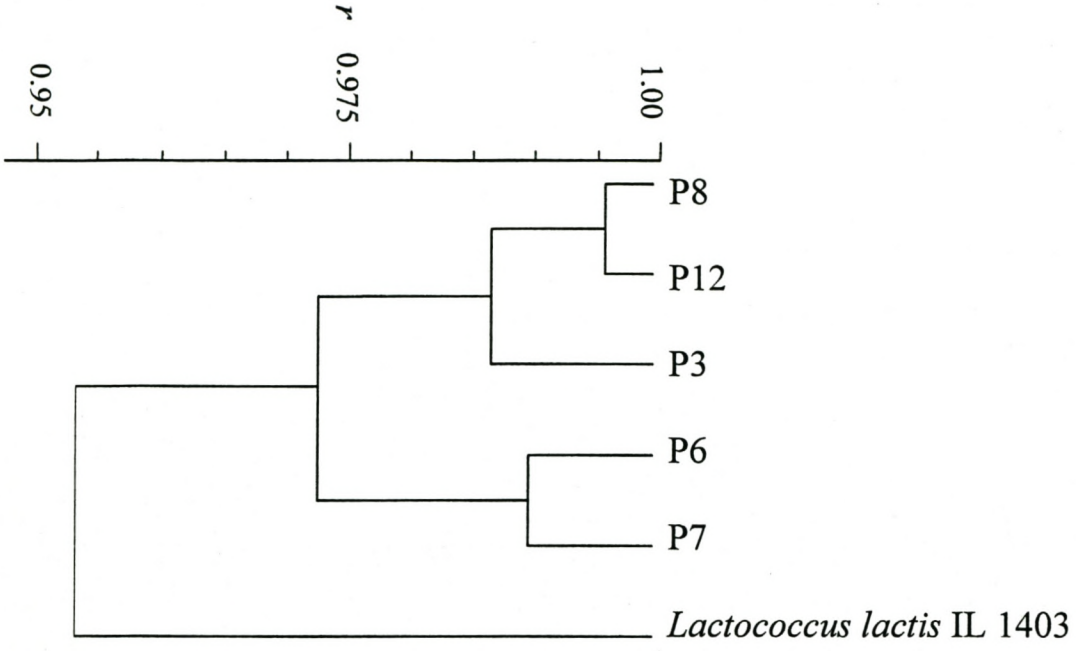

Fig. 5. Dendrogram showing the clustering of *Lactococcus* spp. obtained by numerical analysis of total soluble cell protein patterns. Clustering was by UPGMA. All strains were isolated from the Prisma cultivar.

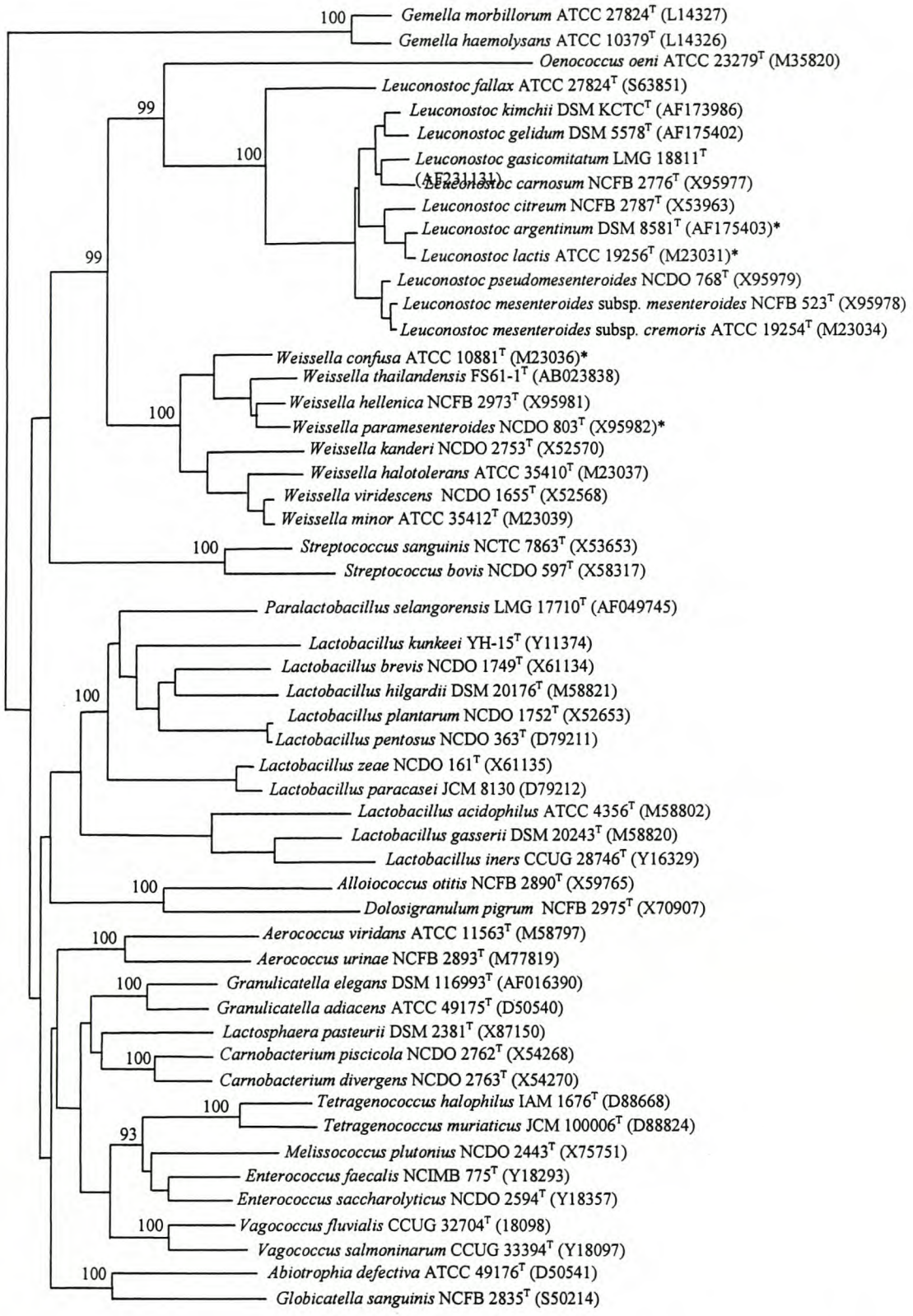

Fig. 6. Unrooted tree showing the phylogenetic relationships of lactic acid bacteria isolated from barley and malt. The positions where the malt strains grouped is indicated by an asteriks. Strains P28, C29, P13, P26, P18, C56, C64, P11, P14, C67 and C68 grouped with *Le. argentinum* DSM 8581^T; strains C52, P10, P48, P1 and P5 grouped with *Le. lactis* ATCC 19256^T; strains P39, P20, P4, P9 and C55 grouped with *W. confusa* ATCC 10881^T; and strains P23, P35, P19 and P25 with *W. parameseneroides* NCDO 803^T.

Table 1

Cell counts of LAB (cfu/g) in Clipper and Prisma

Sample	Clipper	Prisma
Barley before Steep	1.2×10^3	7.6×10^4
Water (first steep)	9.0×10^5	5.5×10^5
Barley (first dry stand)	4.3×10^5	4.4×10^5
Water (second steep)	6.8×10^7	2.4×10^7
Barley (during second steep)	4.0×10^7	2.4×10^6
Barley (second dry stand)	2.4×10^7	8.4×10^7
Green malt (GV[a] day 1)	3.1×10^8	2.5×10^7
Green malt (GV day 2)	1.5×10^7	1.9×10^7
Green malt (GV day 3)	3.7×10^7	3.2×10^7
Kilned malt	1.5×10^4	6.9×10^4

[a]GV = germination vessel

Table 2
Grouping of lactic acid bacteria isolated from Prisma and Clipper malt based on selected carbohydrate fermentation reactions[a]

Characteristic	HE-F cocci resembling *Leuconostoc* spp.		HE-F (Group III) rods resembling *Lactobacillus* spp.		HO-F rods resembling *Lactobacillus* spp.		HO-F cocci resembling *Lactococcus* spp.
	13 strains	25 strains	13 strains	4 strains	2 strains	5 strains	5 strains
Amygdalin	-	+	+	(+)	+	+	d
Arabinose	-	d	-	d	d	-	-
Arbutin	-	-	ND	-	+	+	+
Cellobiose	-	+	+	(+)	+	+	+
Esculin	-	-	+	d	+	+	+
Fructose	+	d	+	+	+	+	+
Galactose	d	+	+	+	+	+	+
β-Gentibiose	d	d	d	d	+	-	+
Lactose	+	+	-	(+)	+	d	+
Maltose	+	+	+	+	+	d	+
Mannitol	-	+	-	(+)	+	+	+
Mannose	d	+	+	+	+	+	+
Melibiose	d	d	-	+	-	-	-
Rhamnose	-	-	-	-	+	-	-
Ribose	d	+	+	d	+	-	+
Salicin	d	-	+	-	+	+	+
Sucrose	+	+	+	+	+	-	d
Sorbitol	-	-	-	-	+	-	-
Tagatose	d	+	d	d	+	+	-
Trehalose	-	d	-	+	+	+	+
Turanose	d	-	d	d	+	d	-
Xylose	-	d	+	d	-	-	-

[a]HE-F, heterofermentative (CO_2 production from glucose); HO-F, homofermentative (no CO_2 production from glucose); +, 90% or more of the strains are positive; (+), delayed positive reaction; -, 90% or more of the strains are negative; d, 11 to 98% of the strains are positive; ND, not determined.

Table 3

Identification of LAB based on numerical analyses of total soluble cell protein patterns, RAPD-PCR banding patterns and 16S rRNA sequencing.

	IDENTIFICATION BASED ON:		
Isolate	PAGE[a]	RAPD-PCR[b]	16S rRNA[c]
From Figs. 1 and 2:			
C50	*Leuconostoc lactis*	*Le. lactis*	
C41	"		
C52	"		*Le. lactis*
C43	"		
P10	"		*Le. lactis*
P38	"	*Le. lactis*	
P48	"		*Le. lactis*
C42	"		
C47	"	*Le. lactis*	
P40	"		
P1	"	*Le. lactis*	*Le. lactis*
P5	"	"	"
P27	"		
P21	*Leuconostoc argentinum*	*Le. argentinum*	
P28	"		*Le. argentinum*
C29	"		"
P24	"	*Le. argentinum*	
P13	"		*Le. argentinum*
P33	"		
P26	"		*Le. argentinum*
C37	"	*Le. argentinum*	
P18	"		*Le. argentinum*
C65	"		
C61	"		
C56	"		*Le. argentinum*
C62	"		
C63	"	*Le. argentinum*	
C64	"		*Le. argentinum*
C60	"	*Le. argentinum*	
C58	"		
C66	"		
C31	"		
P11	"		*Le. argentinum*
P14	"		"
C67	"		"
C68	"		"
P17	"	*Le. argentinum*	
P 2	"		

Table 3 (continued)

Isolate	IDENTIFICATION BASED ON: PAGE[a]	RAPD-PCR[b]	16S rRNA[c]
From Fig. 3:			
P39	*Weissella confusa*		*W. confusa*
C65	"		
P20	"		*W. confusa*
P32	"		
C34	"		
P15	"		
P4	"		*W. confusa*
P9	"		"
C54	"		
C55	"		*W. confusa*
C53	"		
C57	"		
C59	"		
P23	*Weissella paramesenteroides*		*W. paramesenteroides*
P35	"		"
P19	"		"
P25	"		"
From Fig. 4:			
C36	*Lactobacillus rhamnosus*		
C16	"		
C44	*Lactobacillus casei*		
C45	"		
C46	"		
C49	"		
C51	"		
From Fig. 5:			
P 8	*Lactococcus lactis*		
P12	"		
P3	"		
P6	"		
P7	"		

[a]Grouping based on numerical analysis of total soluble cell protein patterns (Figs. 1, 3, 4 and 5).

[b]Grouping based on numerical analysis of RAPD-PCR banding patterns (Fig. 2).

[c]From Fig. 6.

EFFECT OF BACTERIA ON THE FERMENTING ABILITY OF BREWERS YEAST

Effect of Bacteria on the Fermenting Ability of Brewer's Yeast

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Mixed cultures of *Lactobacillus casei, Lactobacillus rhamnosus, Weissella confusa* and *Weissella paramesenteroides*; *Leuconostoc lactis, Leuconostoc argentinum* and *Lactococcus lactis*; and *Citrobacter*, *Entrobacter*, *Proteus* and *Pantoea* spp., previously isolated from Clipper and Prisma malt, were co-inoculated in wort with a brewer's yeast strain of *Saccharomyces cerevisiae.* Changes in gravity, pH, yeast- and bacterial cell numbers and volatile aroma compounds were determined over 10 days of fermentation. All fermentations were done in Clipper and Prisma wort, respectively. The presence of lactic acid bacteria (LAB) and Gram-negative bacteria did not change the gravity of the fermenting wort. The pH profiles recorded for Prisma and Clipper wort fermented in the presence of Gram-negative bacteria were very similar to that recorded in the absence of bacteria. The end pH of Prisma wort was slighty lower (ca. 4.2) when fermented in the presence of lactic acid bacteria. Similar results were recorded in Clipper wort, except that the end pH was 4.5 when fermented with a combination of *Lactobacillus* and *Weissella* spp. and 4.3 in the presence of a combination of *Leuconostoc* and *Lactococcus* spp. Slightly higher yeast cell numbers were recorded in Clipper wort after two days (ca. 9 x 10^7 tcc/ml) compared to Prisma wort over the same period (ca. 8 x 10^7 tcc/ml). After the first week of fermentation the lowest yeast numbers were recorded in the presence of a combination of *Lactobacillus* and *Weissella* spp. in both worts, with little variation towards the end of fermentation. The LAB count increased from ca. 10^7 to ca. 10^9 in the first two days of fermentation, after which the cell numbers more-or-less stabilised for the remainder of the fermentation period. The Gram-negative bacteria decreased from ca. 10^7 to almost zero in Prisma wort over 10 days, whilst cell numbers of 10^3 were recorded in Clipper wort over the same period.

Key Words: *Bacteria, yeast, fermentation, and volatile aroma compounds*

INTRODUCTION

Beer is a complex mixture with more than 400 different flavour compounds which, in addition contains volatile and non-volatile constituents [5,6]. Some of the constituents of beer are derived from the raw materials and survive the brewing process unchanged. Others are the result of chemical and biochemical transformations of the raw materials during malting, mashing and boiling, fermentation and conditioning [7,8]. Different beers and lagers contain different proportions of the same compounds rather than novel constituents. Changes in wort composition or in process conditions may affect yeast growth and cause changes in the production of metabolic by-products that may contribute to beer flavour. Microorganisms other than yeast often produce metabolites that may render the beer organoleptically unacceptable [7,10]. Wort derived from different malt varieties also produce beer with different complexities [16].

The role that microorganisms play in the aroma and texture of beer has always been disputable [7,11]. Certain brewers advocate the elimination of all microorganisms, except brewer's yeast from the brewing process, whereas others believe some microorganisms may contribute to the distinctive and desirable flavours in beer [9,17]. As with wines and ciders, outstanding flavours in beer are often associated with the metabolic activities of a mixed microbial population [10,12,15].

Pathogenic microorganisms fail to grow in beer [7] and the number of bacterial genera usually encountered in brewing is small. The Gram-positive bacteria usually comprises *Lactobacillus* spp., *Pediococcus* spp. and *Leuconostoc* spp., whilst the Gram-negative genera consists of *Acetomonas* spp., *Acetobacter* spp., *Zymomonas* spp., *Enterobacteriaceae* and *Pectinatus* spp. [7, 8, 10]. These bacteria cause turbidity, acid formation and production of off-flavours [7]. The acids may induce premature yeast flocculation, interfere with yeast metabolism and many are flavour-active [5]. Some strains produce extracellular slime, which gives rise to 'ropey' beer. Many of these bacteria, especially certain Gram-negative genera, are inhibited during the fermentation process and die as the pH decreases to below 4.4 and the alcohol content rises above 2.0% (v/v) [4,5,14].

Wort composition, the yeast strain employed and the conditions established at the start of fermentation play a key role in determining beer flavour and aroma. Flavour-active by-products of yeast metabolism produced during fermentation include organic and fatty acids, higher

alcohols, esters, carbonyls and sulphur compounds. The most important organic acids are pyruvate, succinate, citrate, malate and acetate, while the most important higher alcohols, in terms of flavour, include *n*-propanol, isobutanol, 2-methyl-1-butanol and 3-methyl-1-butanol [3]. Esters are important flavour components that impart flowery and fruit-like flavours and aromas to beers. Their presence is desirable at appropriate concentrations, but failure to control fermentation can result in unacceptable ester levels. The organoleptically important esters include ethyl acetate, isoamyl acetate, isobutyl acetate, ethyl caproate and 2-phenyl acetate, while diacetyl and acetaldehyde are the carbonyls that notably influence beer flavour [3]. According to Hough et al. [7] the seven most important volatile constituents of beer include *n*-propanol, isobutanol (2-methyl propanol), 2-methyl butanol, isoamyl alcohol (3-methyl butanol), β-phenyl ethanol, ethyl acetate and isoamyl acetate. The non-volatile constituents include inorganic salts, sugars, amino acids, nucleotides, polyphenols, hop resins, and other macromolecules [3,7,8]. The volatile compounds responsible for the aroma and bouquet of beer can be resolved by gas-liquid chromatography (GLC) and further identified by mass spectrometry [4,13].

In this study, we investigated the effect of a combination of different bacterial combinations on the fermenting ability of the brewer's yeast *Saccharomyces cerevisiae* SAB 05, in wort derived from malt of the barley cultivars Prisma (imported barley) and Clipper (local barley). Special emphasis was given to the yeast and bacterial counts, the changes in gravity and pH throughout fermentation and the major volatile aroma constituents present at different stages of each fermentation.

MATERIALS AND METHODS

Microorganisms

S. cerevisiae strain SAB 05, used for lager brewing by the South African Breweries, was used in the fermentation experiments. The bacterial strains were previously isolated from the malting process of two barley cultivars (Clipper and Prisma), malted at Southern Associated Maltsters, Caledon, South Africa. The lactic acid bacteria (LAB) were identified as *Weissella confusa*, *Weissella paramesenteroides*, *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Lactococcus lactis*,

Leuconostoc argentinum and *Leuconostoc lactis* by biochemical, phenotypical and genotypical methods [2]. The Gram-negative bacteria were identified as members of the genera *Enterobacter*, *Citrobacter*, *Pantoea* and *Proteus*, according to the API 20E system (bioMerieux, Marcy l'Etoile, France).

Media and growth conditions

The yeast was grown to approx. 10^6 cfu/ml in Clipper and Prisma wort, respectively. The LAB were inoculated into 10 ml MRS broth (Biolab, Biolab Diagnostics, Midrand, South Africa) and incubated at 30°C for 24h. The Gram-negative bacteria were inoculated into 10 ml Nutrient broth (Biolab) and incubated at 37°C. At cell numbers of 10^7 cfu/ml, bacteria belonging to the same genus were pooled in sterile flasks, centrifuged at 14300 xg for 30 min and the pellet resuspended in sterile distilled water to yield a final concentration of 10^7 cfu/ml.

Fermentation experiments

Clipper and Prisma malt, malted at Southern Associated Maltsters, Caledon, South Africa, were used to prepare the wort. Mashing was performed according to ANALYTICA-EBC [1]. The fermentation experiments were performed at 11°C for 10 days, in 2L European Brewing Convention (EBC) tall tubes at Southern Associated Maltsters, Caledon, South Africa. The experiments for gravity- and pH changes, yeast counts and bacterial counts included a control with only yeast and no bacteria, while the other fermentations had mixtures of *Lactobacillus* and *Weissella* spp.; *Leuconostoc* and *Lactococcus* spp.; or Gram-negative bacteria and yeast. Fermentations for the aroma profiles included a control with only yeast, whilst the other fermentations contained *Lactobacillus* spp., *Weissella* spp., *Leuconostoc* spp., *Lactococcus* spp. or Gram-negative bacteria, respectively, together with yeast.

Sampling

Samples for yeast- and bacteria counts, gravity and pH were drawn every 24h throughout the fermentation. Samples for volatile aroma components were done on days 1, 5, 8 and 10 of each fermentation. Samples were collected aseptically by washing the sampling taps of the EBC tubes with 70% (v/v) ethanol, flushing the taps before sampling and washing the taps with ethanol again after sampling.

Determination of yeast and bacterial counts

Yeast counts were done on a haemocytometer (Model 105/04, Superior, Germany). Bacterial counts were done by plating onto MRS Agar (Biolab).

pH and gravity determinations

Gravity- and pH determinations were done by centrifuging 5 ml of each sample at 1090 xg for 5 minutes, where-after the supernatant were filtered through 1.1 mm Whatman filterpaper (Merck) into 5 ml gravity sample vials (Chromatography Research Supplies, USA). The pH-readings were determined using a pH-meter (Crison, Barcelona, Spain), while the gravity readings were measured using the Anton Paar DMA 55 density meter (Anton Paar, Graz, Australia).

Volatile aroma tests

Samples for volatile aroma compound determinations were obtained by centrifuging 50 ml of each sample at 1590 xg at 4°C for 10min., whereafter the supernatant was filtered through a double-layer of 1.1 mm Whatman filterpaper. The filtered samples were stored at -80°C. The volatile aroma compounds were determined with a liquid-liquid extraction and gas chromatography method, as described by Prior et al. [13].

RESULTS AND DISCUSSION

Changes in gravity, pH, yeast counts and bacterial counts obtained after fermenting Prisma and Clipper wort with *S. cerevisiae* strain SAB 05 and different combinations of bacteria are shown in Fig. 1. The gravity in both malt fermentations was reduced from approx. 16°P at the start of fermentation to approx. 2.5°P after 10 days of fermentation (Fig. 1A and B). No significant variation was recorded between the control fermentation (yeast only) and wort fermented with yeast and bacteria.

The pH changes recorded for the two cultivars are shown in Fig. 1C and D, with the initial pH value recorded for all fermentations being ca. pH 5.4. The end pH of wort fermented in the presence of Gram-negative bacteria remained more-or-less the same as wort fermented without bacteria (Fig. 1C and D). The lowest end pH (ca. 4.2) was recorded in Prisma wort fermented in the presence of LAB (Fig. 1C). Small variations were recorded in Clipper wort, with an end pH of 4.3 in the presence of *Leuconostoc* and *Lactococcus* spp. and 4.5 in the presence of *Lactobacillus* and *Weissella* spp. (Fig. 1D). The decrease in pH recorded when the fermentation

was conducted in the presence of LAB is ascribed to the formation of organic acids such as lactic acid, acetic acid and decanoic acid [7].

Samples drawn for yeast counts in the haemocytometer represents viable and non-viable cells, thus a total cell count (tcc). Comparative studies in wort over 10 days have shown that 95% of the yeast cells counted with the haemocytometer are viable (J. Gleisner, personal communication). The yeast counts in the control fermentations (i.e. in the absence of any bacteria) in Prisma (Fig. 1E) and Clipper (Fig. 1F) wort, were slightly higher than the yeast counts in fermentations containing bacteria, indicating that the bacteria may have a negative influence on yeast growth. Compared to the LAB, the Gram-negative bacteria inhibited yeast growth the least of all (Fig. 1 E and F). Slightly higher yeast cell numbers were recorded in fermented Clipper wort (Fig. 1F) after two days (ca. 9×10^7 tcc/ml) compared to fermented Prisma wort (Fig. 1E) over the same period (ca. 8×10^7 tcc/ml). After the first week of fermentation the lowest yeast numbers were recorded in the presence of a combination of *Lactobacillus* and *Weissella* spp. in both worts, with little variations towards the end of fermentation (Fig. 1E and F). The rapid decrease in the yeast count when growing in combination with LAB can be ascribed to an increase in flocculation of the yeast, the production of organic acids, hence a decrease in pH, or as yet unknown antimicrobial substances produced by LAB. The bacteria may also metabolise some of the essential nutrients the yeast needs for growth.

The LAB cell numbers increased from ca. 10^7 to ca. 10^9 in the first two days of fermentation, followed by cell numbers fluctuating between 5×10^8 cfu/ml and 9.8×10^8 cfu/ml to the end of the fermentation (Fig. 1G and H). The Gram-negative bacteria decreased from 6.8×10^6 cfu/ml at the start of fermentation to almost zero in Prisma wort (Fig. 1G), whilst cell numbers of ca. 10^3 cfu/ml were recorded in Clipper wort (Fig. 1H) over the same period. The fact that the Gram-negative bacterial cell numbers decreased significantly throughout the fermentations confirmed that they cannot survive the fermentation conditions, whilst most of the LAB survive these conditions.

Concluding from the results presented in Fig. 1, small differences were recorded in the pH, yeast numbers and overall bacterial cell numbers during the fermentation of Prisma and Clipper malt,

respectively. Of interest is the slightly better yeast growth recorded in Clipper malt (Fig. 1F). This also co-incided with a slightly higher pH maintained throughout the 10-day fermentation period (Fig. 1D). Although no major differences were recorded in LAB cell numbers in the two malt fermentations, the growth of Gram-negative bacteria was slightly more repressed in fermented Prisma malt (Fig. 1G). It is tempting to speculate that the latter may be due to a more stable LAB growth in Prisma malt (Fig. 1G) compared to the growth variations recorded in Clipper malt (Fig. 1H).

The overall aroma profiles recorded for Prisma and Clipper wort fermented with yeast only (Figs. 2 and 3, respectively) were very similar, except for slightly higher levels of acetic acid and decanoic acid recorded in the Prisma wort (83 mg/l and 4.7 mg/l, respectively). Variations among different fermentation runs were less than 5%.

No major changes were recorded in the levels of volatile aroma compounds produced in the Prisma control fermentation, i.e. yeast only (Fig. 2A) and the same wort fermented in the presence of Gram-negative bacteria (Fig. 2B). However, a slight difference was recorded in the acetoin levels, with 15 mg/l recorded on day 5 in the control fermentation (Fig. 2A), compared to 10 mg/l on day 5 in the presence of Gram-negative bacteria, with no acetoin detected on day 8 (Fig. 2B). Similar results were recorded for Clipper wort fermented in the absence and presence of Gram-negative bacteria (Fig. 3A and B, respectively). Of interest, however, is the decrease of decanoic acid from 6.5 mg/l in the control fermentation (Fig. 3A) to 2.5 mg/l when fermented in the presence of Gram-negative bacteria (Fig. 3B). The fact that the Gram-negative bacteria had no significant effect on the levels of the volatile aroma compounds produced during the fermentation of Prisma and Clipper wort could be ascribed to the rapid decrease in the cell counts recorded (Fig. 1G and H).

Prisma malt fermented with *Lactobacillus* spp. produced high levels of acetic acid (174 mg/l) during the first 5 days of fermentation (Fig. 2C), which subsequently decreased to levels corresponding to the control fermentation (75 mg/l, Fig. 2A). Similar, but lower, levels of acetic acid production were recorded in Clipper fermentations (Fig. 3A and C). The aroma profiles of *Weissella* spp. in the two different malt fermentations (Figs. 2D and 3D) were very similar to that recorded for *Lactobacillus* spp. Variations recorded in acetic acid levels are reflected in the

growth of the yeast, with better yeast growth in the Prisma wort fermentation (Fig. 1D). Concluded from the decrease in pH and acetic acid production, higher levels of the acid were produced after the second day of fermentation. A further point of interest was the abnormal high levels of 2-phenyl ethane recorded on days 5 and 8 of fermented Prisma wort (Fig. 2C and D) and on days 5, 8 and 10 of fermented Clipper wort (Fig. 3C and D), compared to the relatively low concentrations recorded for the two control fermentations (Figs. 2A and 3A, respectively). The effect of 2-phenyl ethane on yeast growth is not known and merits further investigation.

Leuconostoc spp. had a much greater influence on the aromatic composition of fermented wort, with much more clear variations between Prisma and Clipper (Figs. 2E and 3E). In fermented Prisma wort acetic acid concentrations as high as 267 mg/l were obtained on day 8 (Fig. 2E). This could contribute to the low pH recorded in fermented wort containing a combination of *Leuconostoc* and *Lactococcus* spp. (Fig. 1C). Levels of ethyl acetate, iso-amyl alcohol and propanol in the Prisma wort (Fig. 2E) differed from the concentrations in the control fermentation (Fig. 2A). As detected with *Lactobacillus* and *Weissella* spp., the 2-phenyl ethane levels in Prisma also increased, *viz.* 1.0 to 12.8 mg/l (Fig. 2E). In contrast to the already mentioned LAB, Prisma wort fermented with *Leuconostoc* spp. had higher levels of octanoic acid and decanoic acid levels (10.2 mg/l and 7.6 mg/l, respectively, Fig. 3E). Although the aroma profiles of Prisma and Clipper wort fermented with *Leuconostoc* spp. corresponded well (Figs. 2E and 3E), the levels of octanoic and decanoic acid in Clipper wort (Fig. 3E) did not differ much from the concentrations recorded in the control fermentation (Fig. 3A). The relatively low concentration of the latter two fatty acids could be the reason why yeast growth in Clipper wort was slightly better than recorded in Prisma wort.

No major differences were recorded in the aroma profiles of Prisma and Clipper wort fermented in the presence and absence of *Lactococcus* spp. (Figs. 2F and A, and 3F and A, respectively). The biggest difference between the two fermentations was in the production of 2-phenyl ethane.

We have reported on five of the seven principle volatile constituents produced by brewer's yeast, as discussed by Hough et al. [7]. These compounds included the higher alcohols propanol, isobutanol (2-methyl propanol) and isoamyl alcohol (3-methyl butanol), and the esters ethyl acetate and isoamyl acetate. According to Hough et al. [7] and Nykänen and Suomalainen [11]

acceptable levels of propanol in lager beer vary between 5 and 10 mg/l, isobutanol between 6 and 11 mg/l and isoamyl alcohol between 32 and 57 mg/l. The acceptable levels for the two esters range between 8 and 14 mg/l for ethyl acetate and 1.5 and 2 mg/l for isoamyl acetate [7]. The levels of these aroma compounds differ for each yeast strain and the acceptable production levels depend on the specific brand of beer [7,11].

The levels of four of the five principle aroma compounds reported in this study, i.e. propanol, isobutanol (2-methyl propanol), isoamyl alcohol (3-methyl butanol), ethyl acetate and isoamyl acetate, on the tenth day of fermentation in the control fermentations of Clipper (Fig. 3A) and Prisma (Fig. 2A) were in the acceptable concentration range. However, the concentration of ethyl acetate in both control fermentations was higher than the acceptable levels. The same trend was observed in the levels of the five aroma compounds in the fermentations containing yeast and Gram-negative bacteria (Figs. 2B and 3B). The levels of the five principle volatile compounds on the tenth day of fermented wort containing yeast and the different LAB genera (Figs. 2C, D, E, F and 3C, D, E, F) differ from that of the control fermentations (Figs 2A and 3A). Generally, the concentrations of these aroma compounds were lower than in the control fermentations, suggesting that the bacteria may have an effect on the yeast in producing these metabolites. The levels of isoamyl alcohol, propanol and isoamyl acetate on day 10, in all the fermentations containing LAB, are in the acceptable concentration range, while the levels of ethyl acetate are higher than the acceptable levels and isobutanol levels lower.

The levels of almost all the volatile aroma components, especially acetic acid, are higher in Clipper wort than in Prisma wort for the different fermentation conditions. The aroma compounds produced at the highest concentrations in both Clipper and Prisma were ethyl acetate, iso-amyl alcohol, acetic acid and acetoin, with acetic acid being present in the highest concentration in all the fermentations. The high acetic acid concentrations in the fermentations containing LAB was previously described by Nykänen and Suomalainen [11].

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REFERENCES

1. Bishop, L.R., *Analitica-EBC 2nd ed.*, Elsevier Publishing Company, London, 1963, 14.

2. Booysen, C., Dicks, L.M.T., Ackermann, A., Meijering, I., *International Journal of Food Microbiology*, 2001.

3. Boulton, C.A., *Genetic Engineering Reviews*, 1991, **9**, 127.

4. Chen, E. C. H., David, J.J., *Journal of Science Food and Agriculture*, 1974, **25**, 1381.

5. Evans, D. J., Schmedding, D.J.M., Bruinje, A., Heideman, T., King, M., Groesbeek, N.M., *Journal of the Institute of Brewing*, 1999, **105**, 301.

6. Gilliland, R.B., Harrison, A.F., *Journal of Applied Bacteriology*, 1966, **29**, 244.

7. Hough, J.S., Briggs, D.E., Stevens, R., Young, T.W., *Malting and Brewing Science 2nd ed.*, Chapman and Hall, London, 1982, 566.

8. Hough, J., *The Biotechnology of Malting and Brewing*, Cambridge University Press, 1985, 114.

9. King, A., Dickinson, J.R., *Yeast*, 2000, **16**, 499.

10. Martens, H., Iserentant, D., Verachtert, H., *Journal of the Institute of Brewing*, 1997, **103**, 85.

11. Nykänen, L., Suomalainen, H., *Aroma of beer, wine and distilled alcoholic beverages*, D. Reidel publishing company, London, 1983.

12. Pollard, A., Kieser, M.E., Beech, F.W., *Journal of Applied Bacteriology*, 1966, **29**, 253.

13. Prior, B.A., Toh, T.H., Jolly, N., Baccari, C., Mortimer, R.K., *South African Journal of Enology and Viticulture*, 2000, **21**, 92.

14. Rangel, A.O.S.S., Fernandes, S.M.V., *Journal of the Institute of Brewing*, 1998, **104**, 203.

15. Stratford, M., *FEMS Microbiological Letters*, 1996, **136**, 13.

16. Tulej, R.M., *The fermentability of wort derived from various malts*, University of Stellenbosch, South Africa, 1991.

17. Van der Lans, R.G.J.M., van Hamersveld, E.H., Caulet, P.J.C., Luyben, K.C.A.M., *Biotechnology and Bioengineering*, 1998, **57**, 330.

FIG. 1 Results obtained after fermenting Prisma and Clipper wort with yeast and different bacterial combinations. The graphs show the changes in gravity (A, B), pH (C, D), yeast counts (E, F) and bacterial counts (G, H) in Prisma and Clipper wort, respectively. Control, i.e. *Saccharomyces cerevisiae* SAB 05 in the absence of bacteria (■), yeast and Gram-negative bacteria (✳), yeast and a combination of *Lactobacillus* and *Weissella* spp. (Δ), yeast and a combination of *Leuconostoc* and *Lactococcus* spp. (✕).

(tcc/ml = total cell count per milliliter wort)

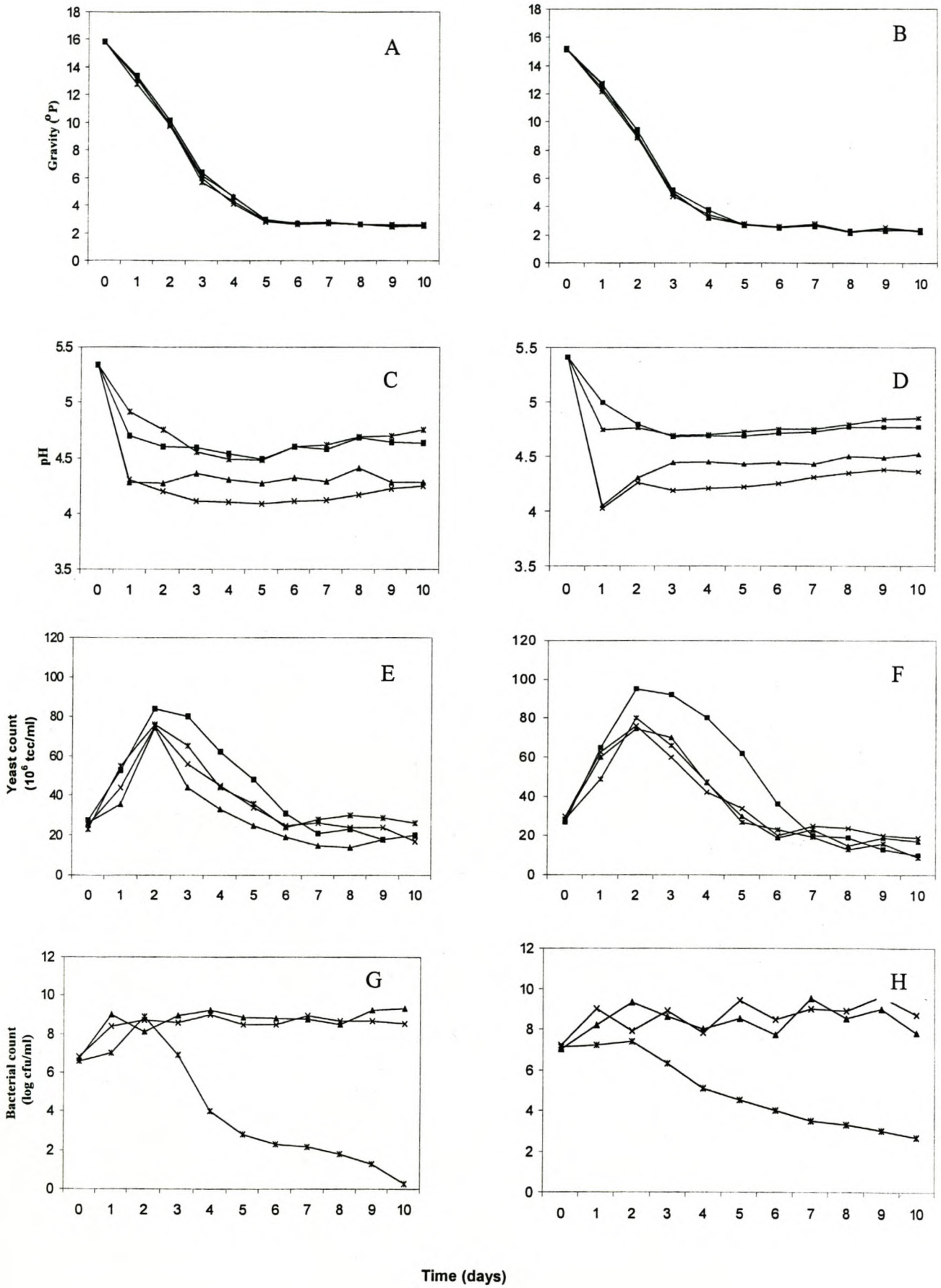
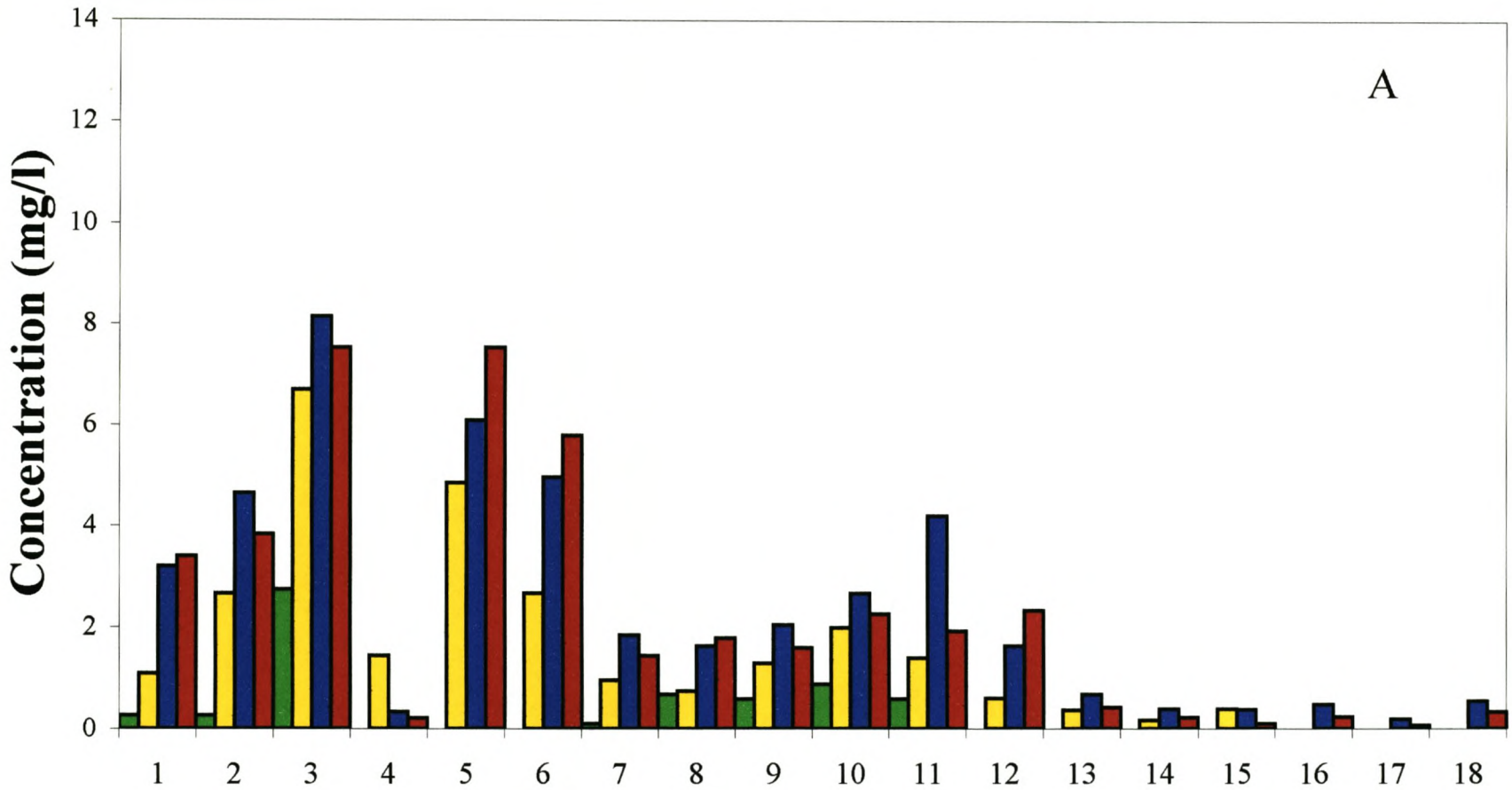


FIG. 2 Levels of volatile aroma compounds detected during the fermentation of Prisma wort, with yeast and different bacteria. Control, i.e. *Saccharomyces cerevisiae* SAB 05 in the absence of bacteria (A), yeast and Gram-negative bacteria (B), yeast and *Lactobacillus* spp. (C), yeast and *Weissella* spp. (D), yeast and *Leuconostoc* spp. (E) and yeast and *Lactococcus* spp. (F). Samples were taken on day 1 (■), day 5 (■), day 8 (■) and day 10 (■).

* = Concentration x 10



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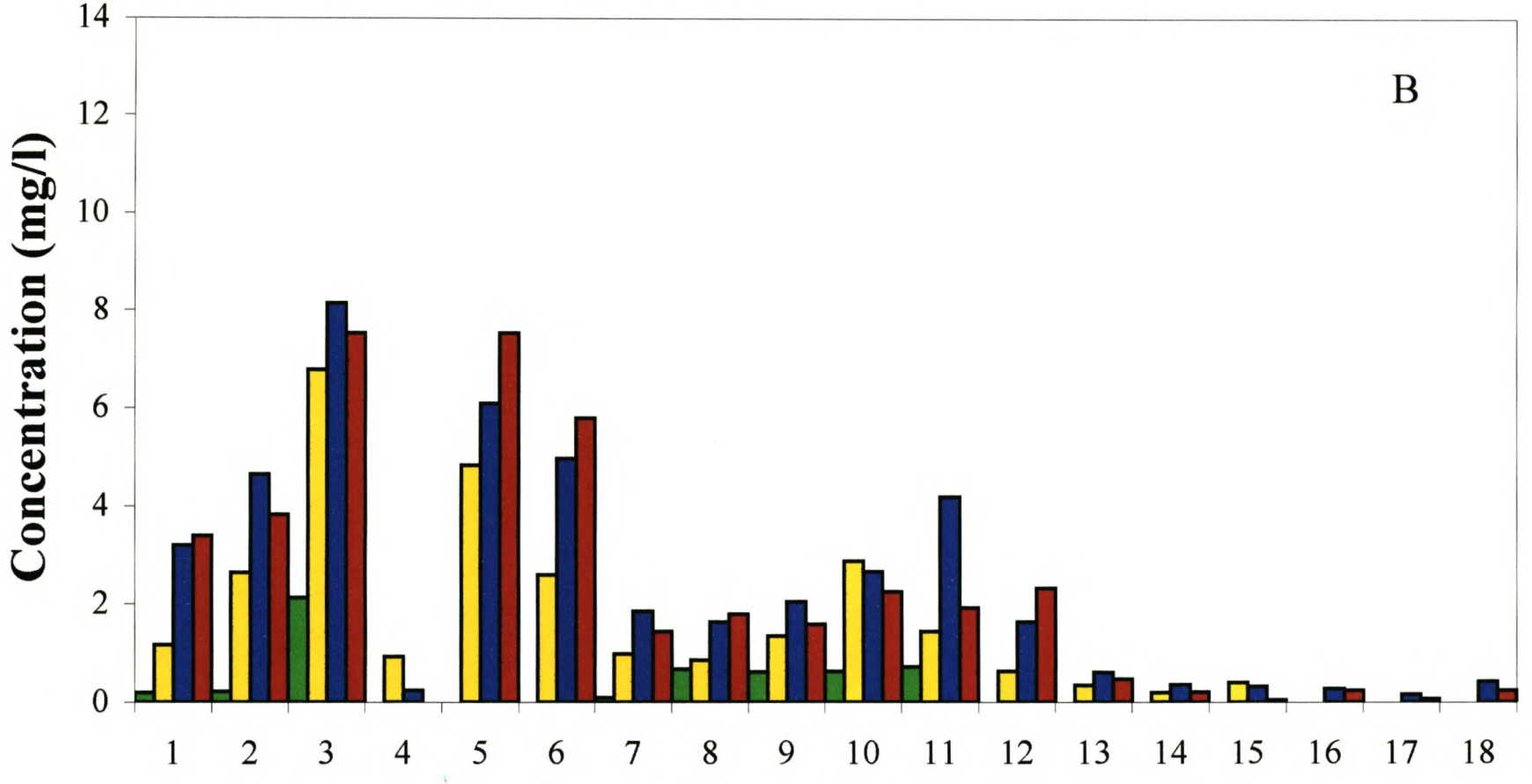

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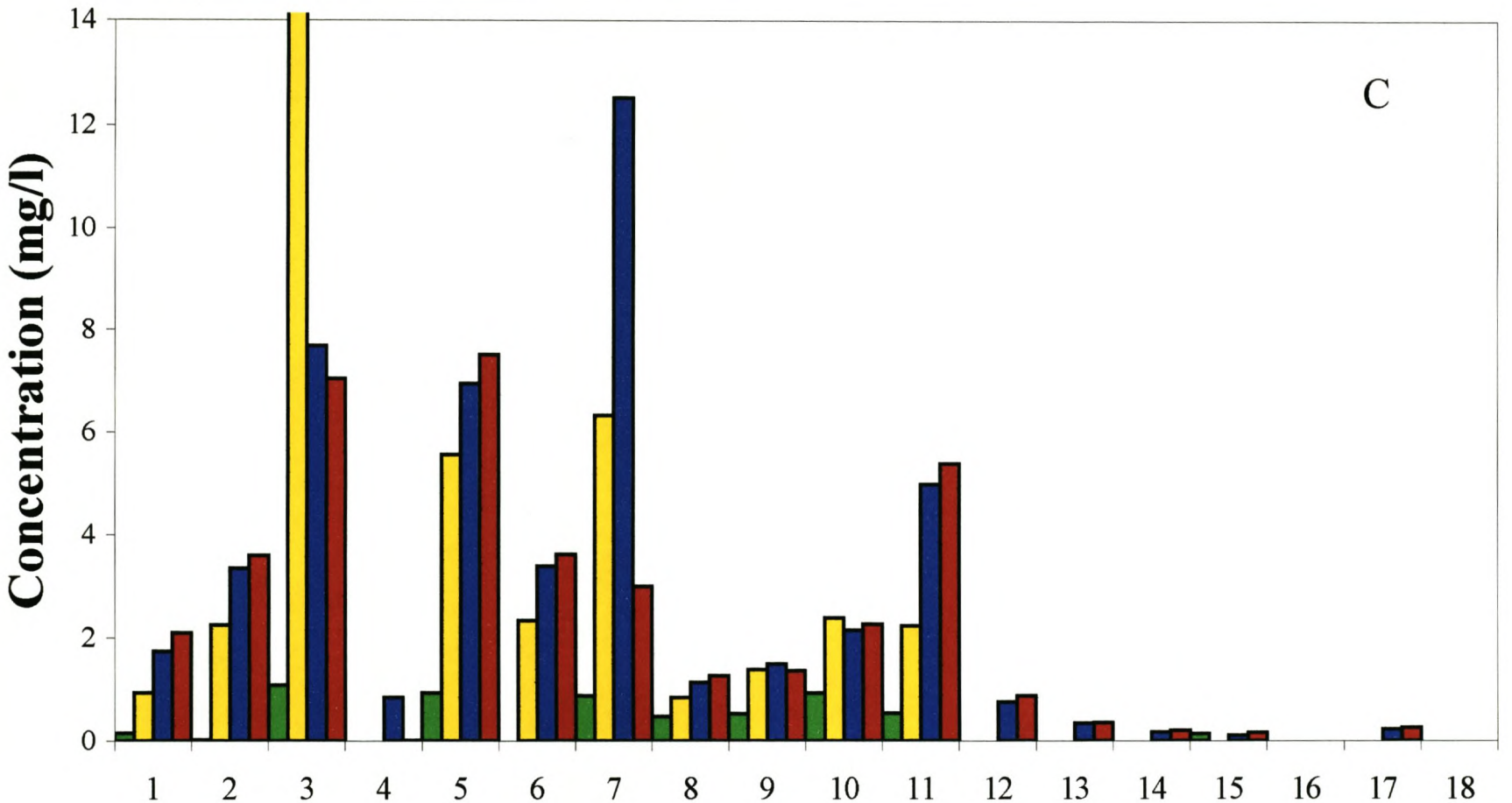

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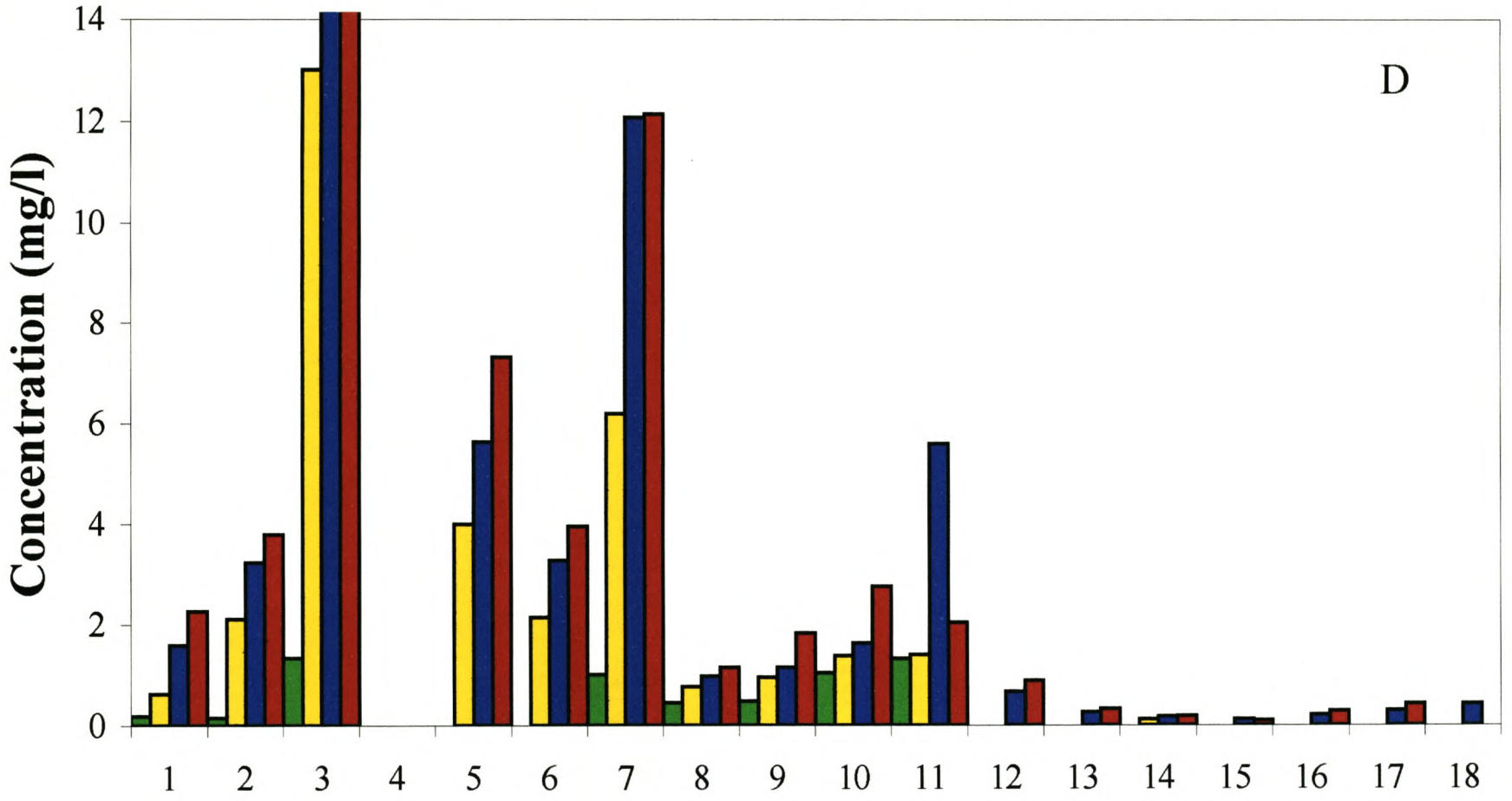

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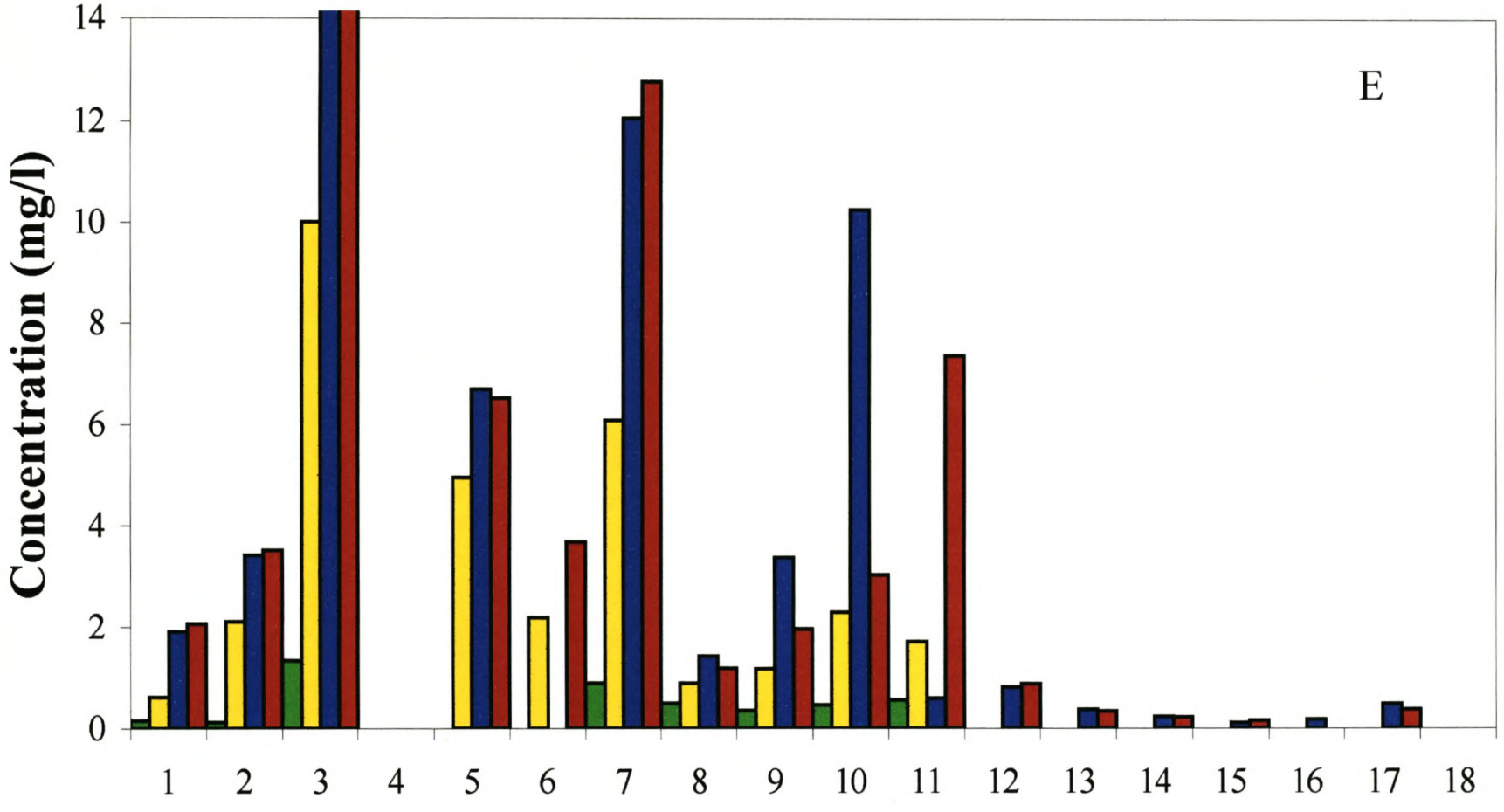

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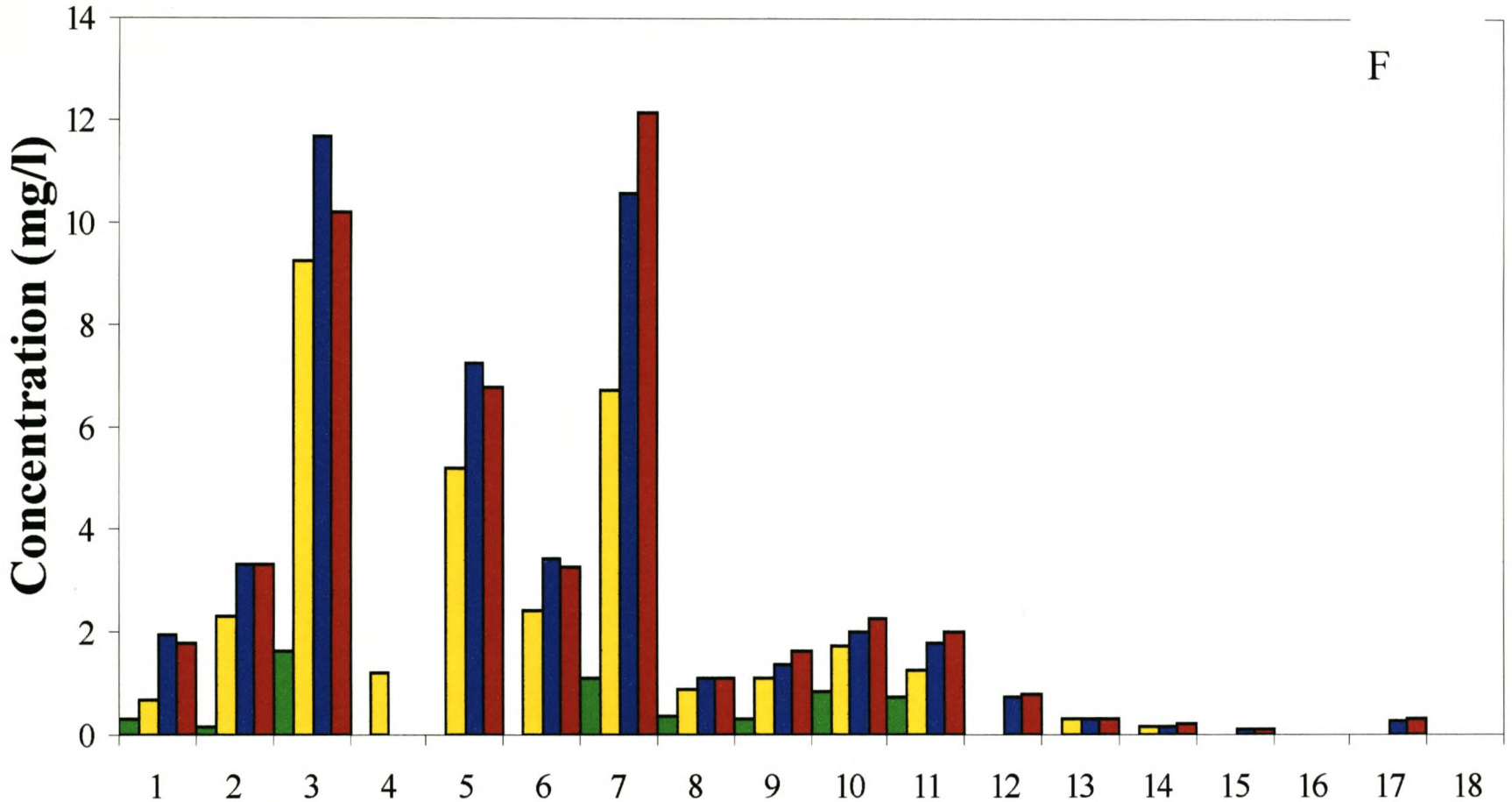

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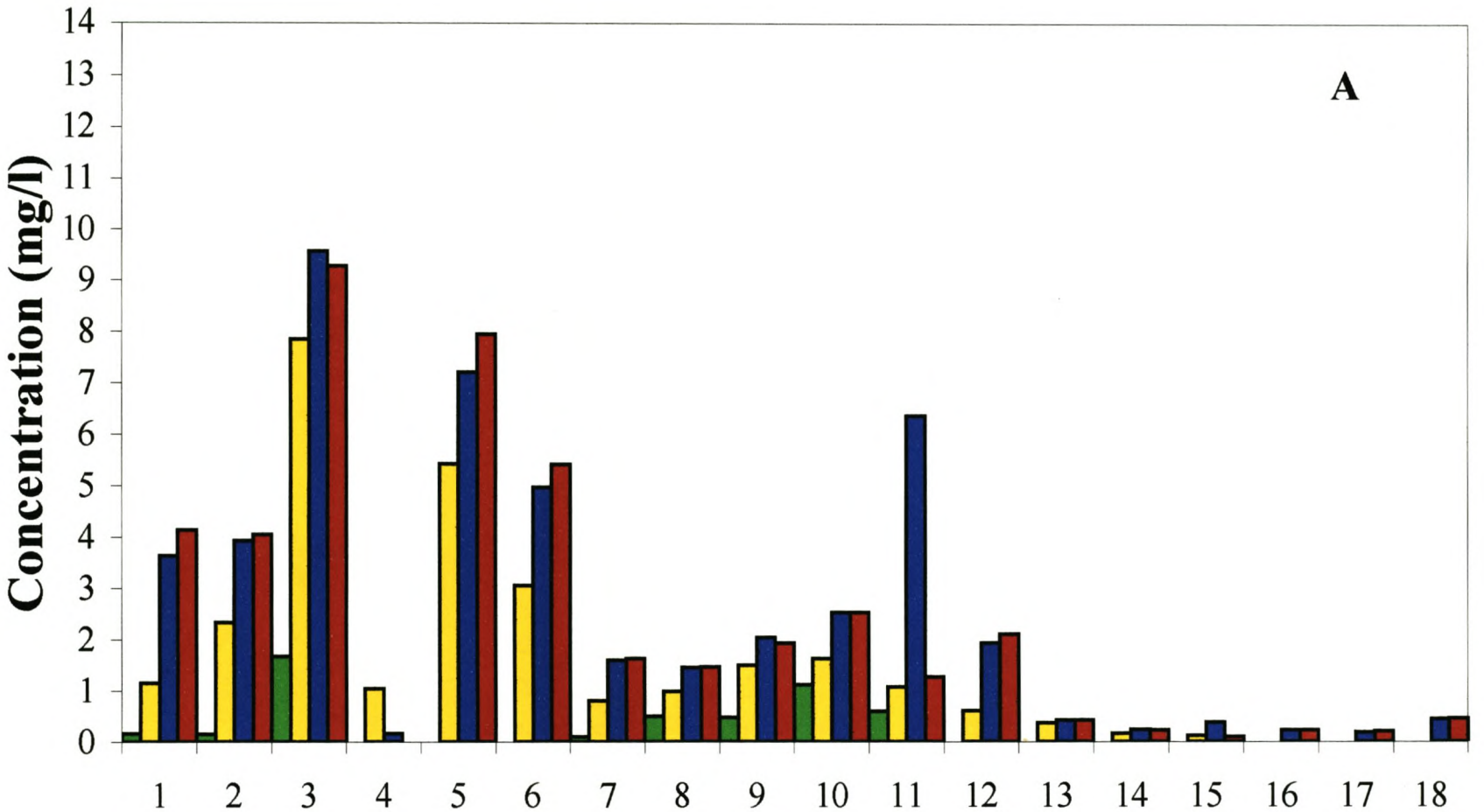
FIG. 3 Levels of volatile aroma compounds detected during the fermentation of Clipper wort, with yeast and different bacteria. Control, i.e. *Saccharomyces cerevisiae* SAB 05 in the absence of bacteria (A), yeast and Gram-negative bacteria (B), yeast and *Lactobacillus* spp. (C), yeast and *Weissella* spp. (D), yeast and *Leuconostoc* spp. (E) and yeast and *Lactococcus* spp. (F). Samples were taken on day 1 (■), day 5 (■), day 8 (■) and day 10 (■).

* = Concentration x 10



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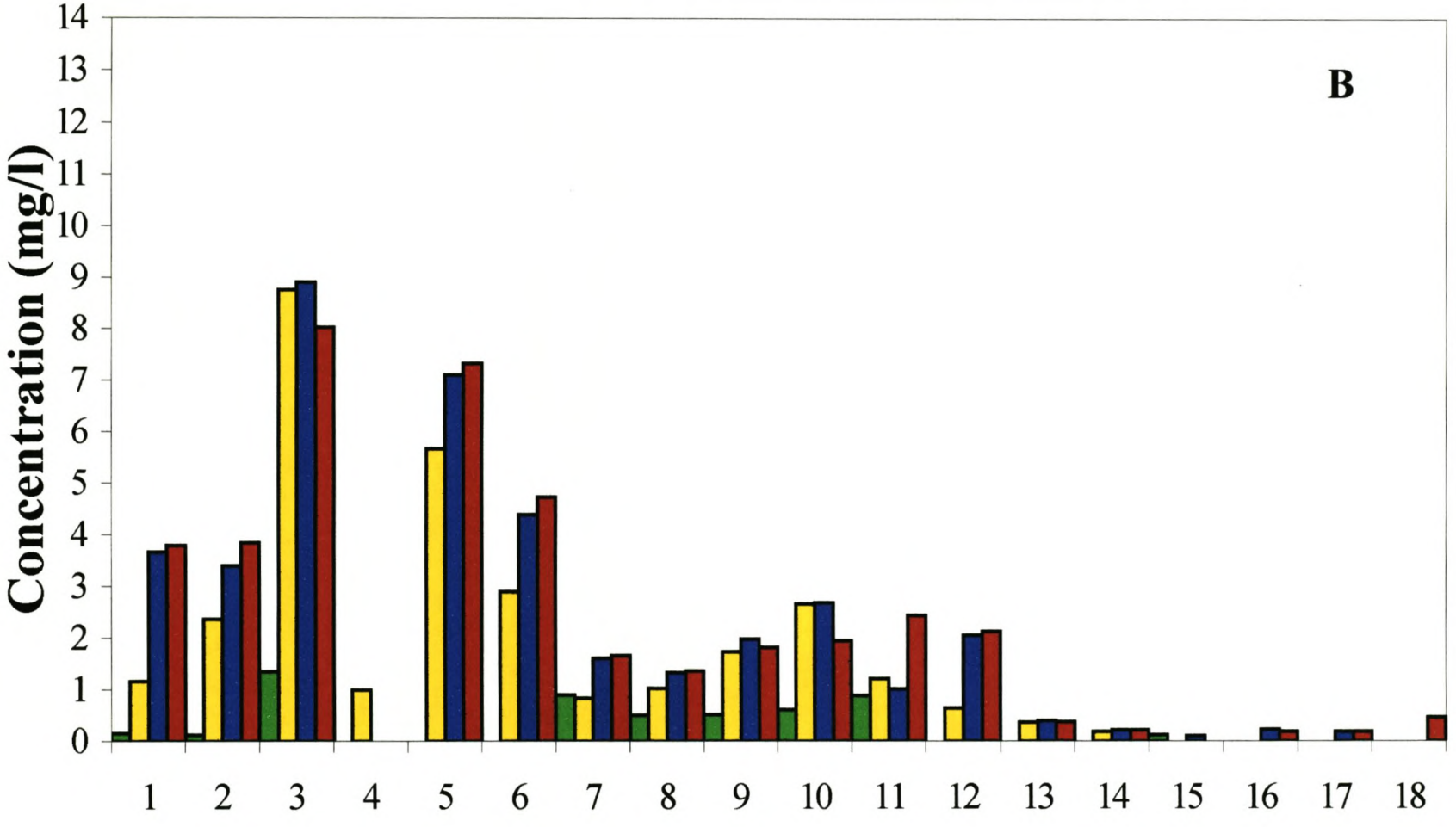

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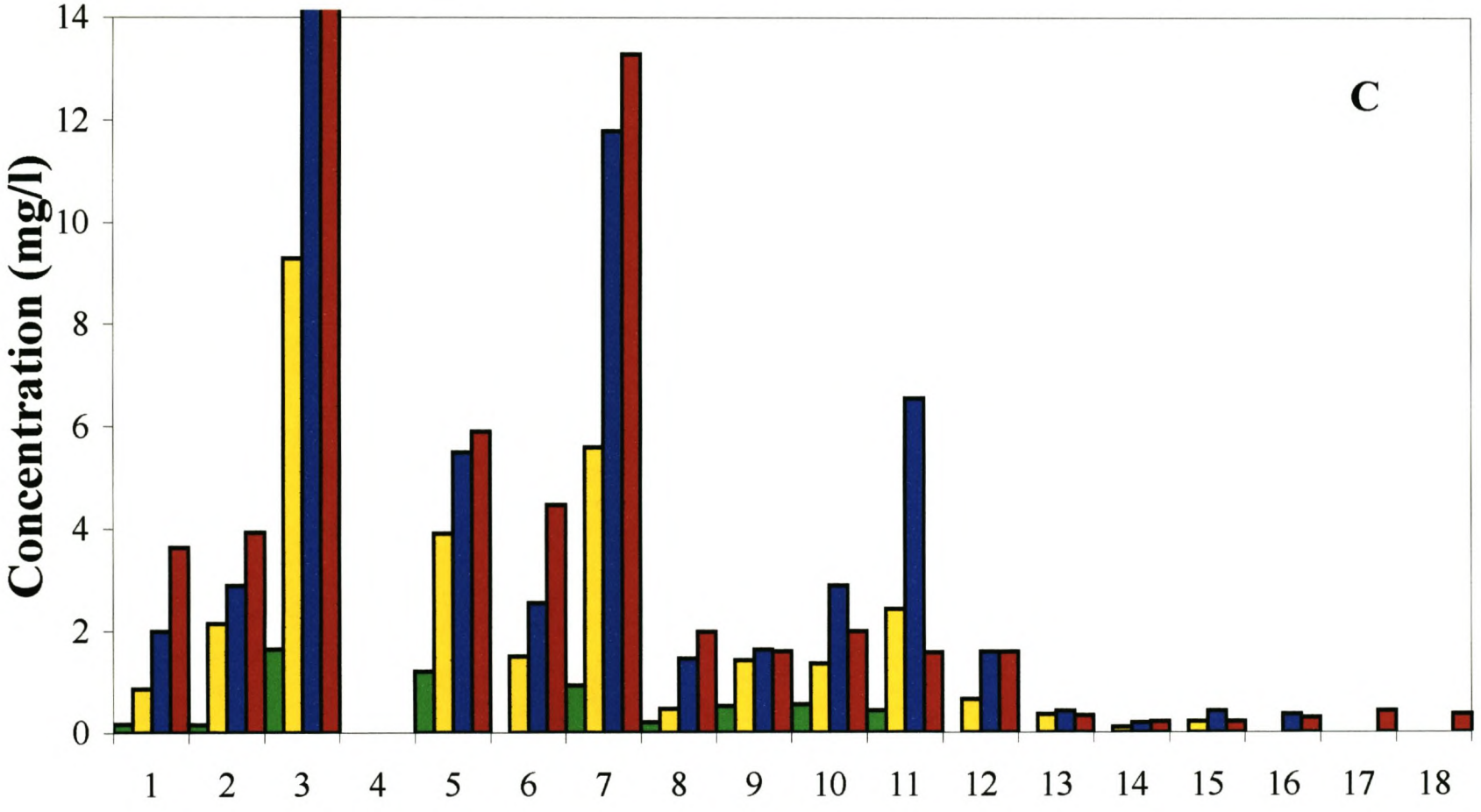

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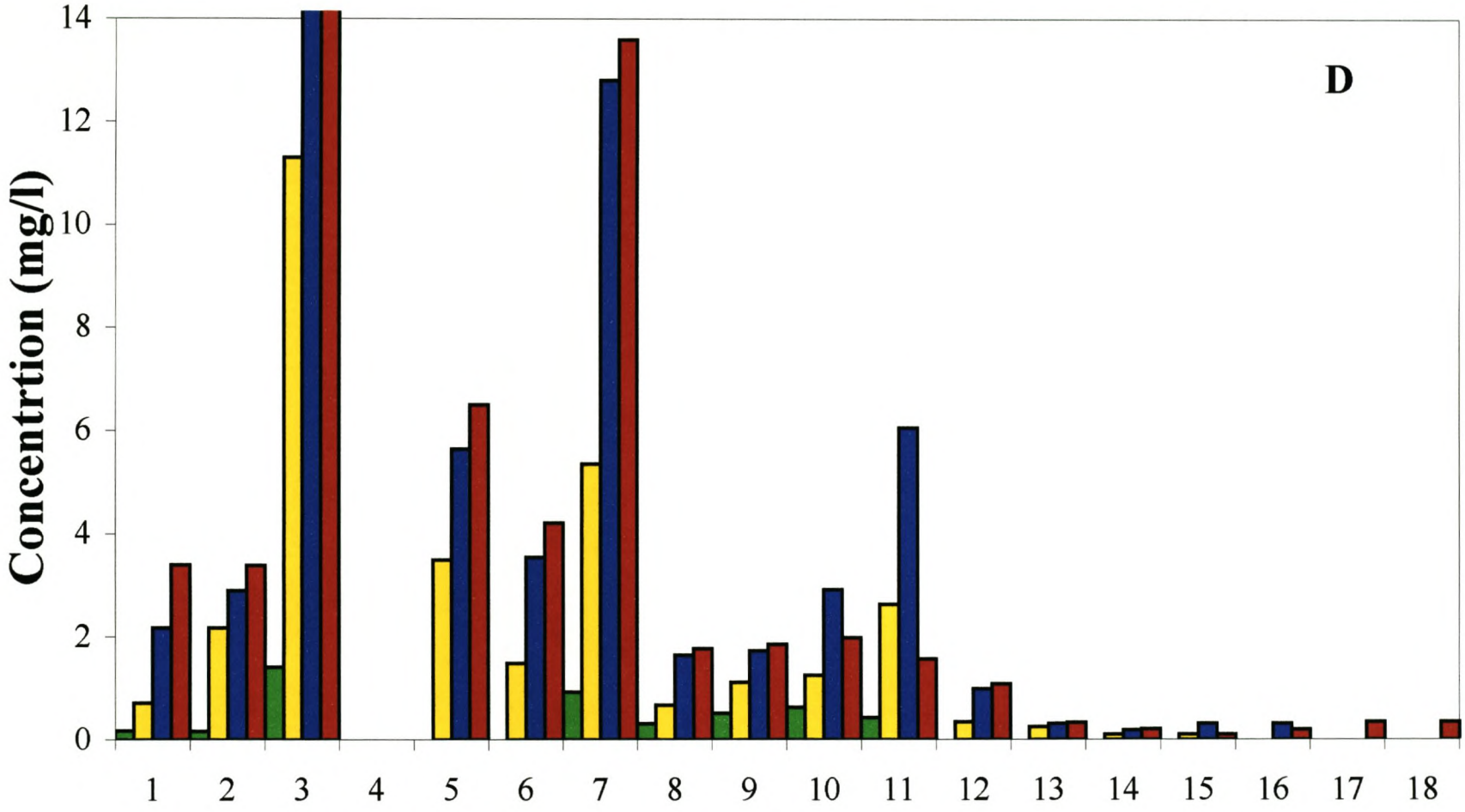

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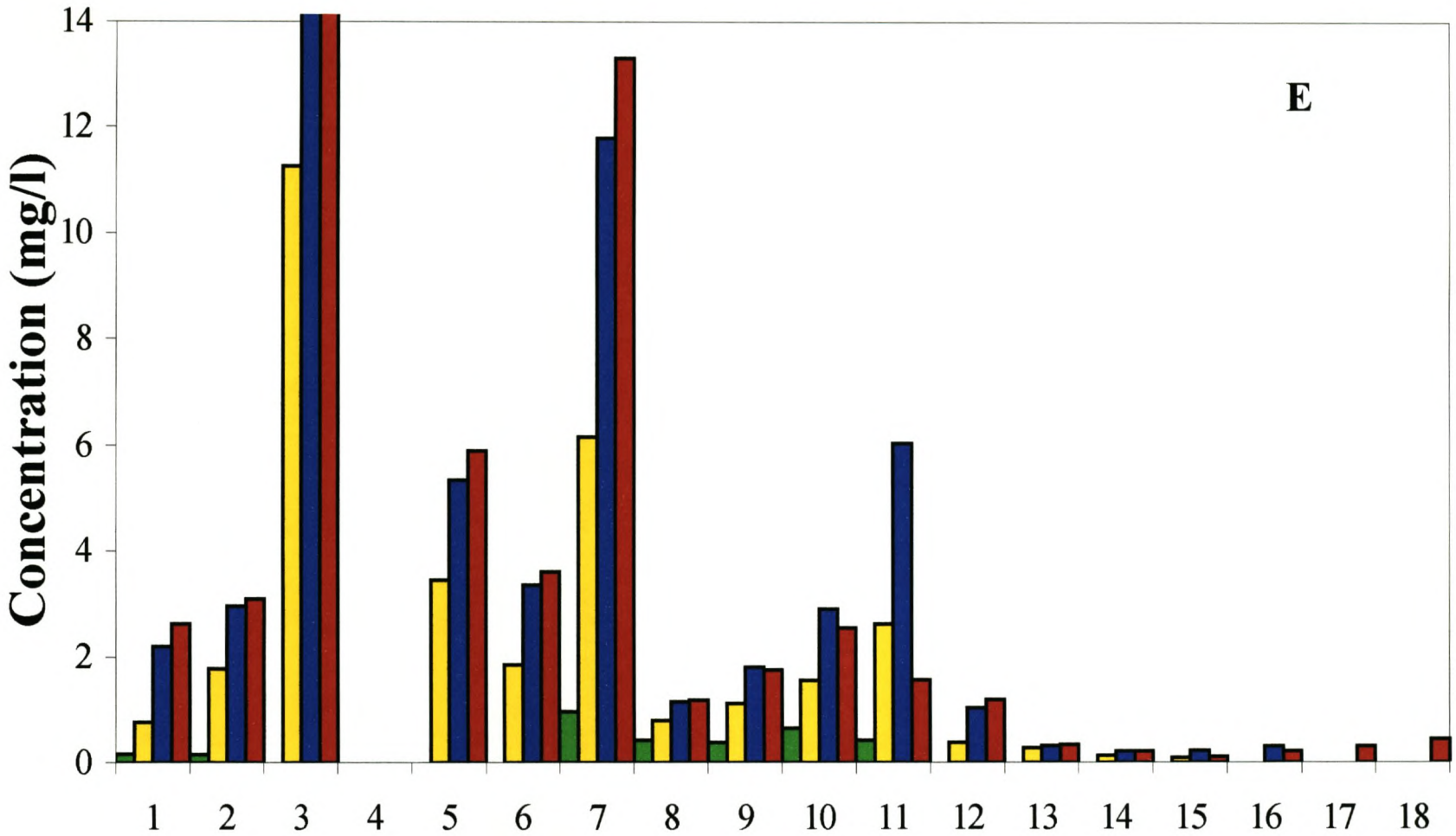

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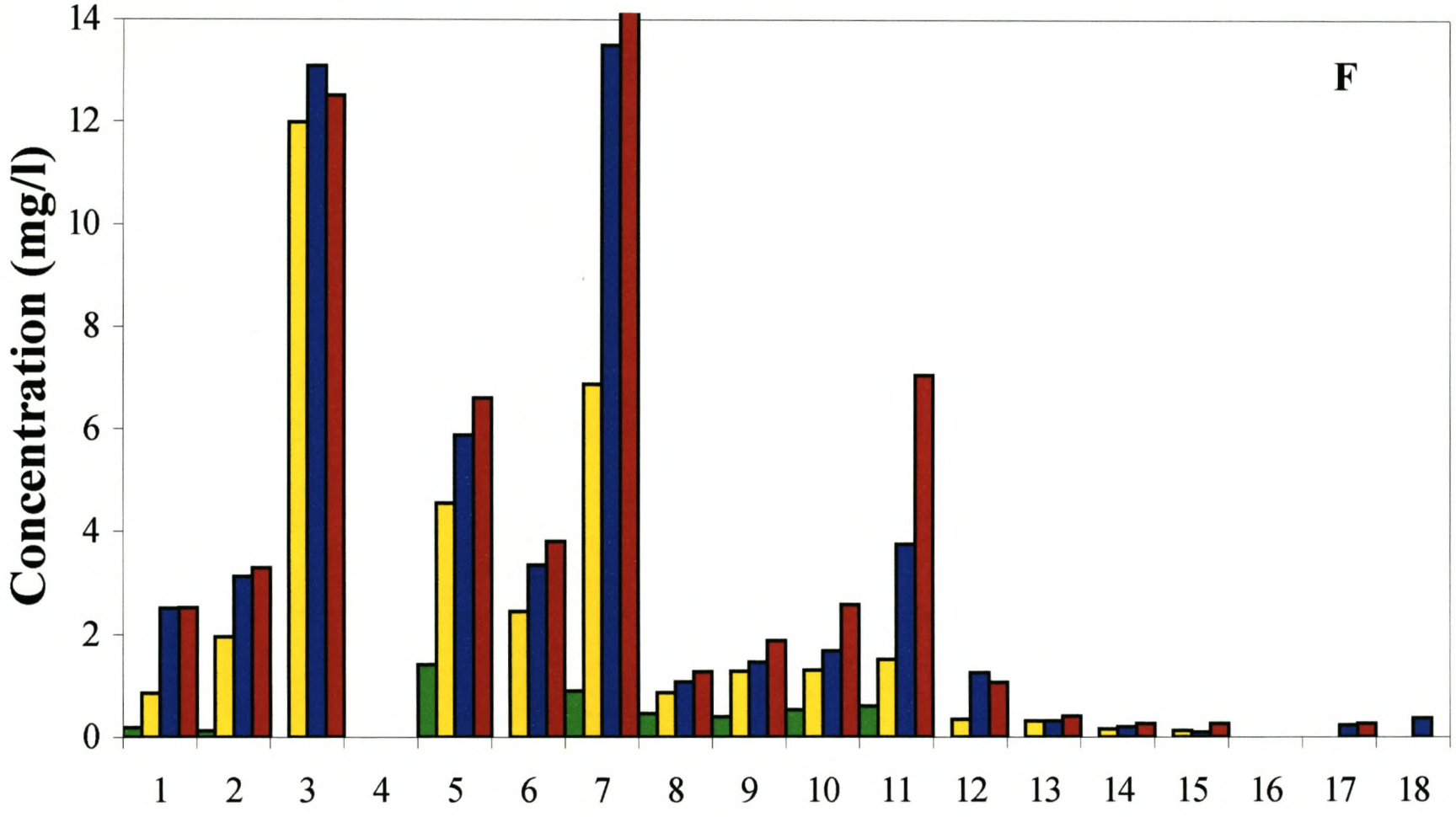

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GENERAL DISCUSSION AND CONCLUSION

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Lactic acid bacteria (LAB) are widespread in nature and are found in habitats with high concentrations of soluble carbohydrates, protein breakdown products, vitamins and a low oxygen tension. The natural habitat of these bacteria is plants, as evident from the many species isolated from vegetables, fruit, silage, dough, wine, beer and other traditional fermented plant material (De Vuyst and Vandamme 1994). Although LAB has GRAS (generally regarded as safe) status they play an important role in the spoilage of processed and fermented foods, and beverages. Examples include souring and off-flavours in meat products, the spoilage of wine, beer, and fruit juices by *Pediococcus*, *Leuconostoc* and *Lactobacillus* spp. An example of beer spoilage includes cloudiness, off-flavours, and the production of exopolymers by *Leuconostoc* ssp. (Aguirre and Collins 1993). Malt has a complex microbial population that changes as the process commences (Peters *et al.* 1988; Kelly and Briggs 1992). High LAB counts on malt cause mash filtration difficulties during brewing. However, LAB are used as starter cultures in the steep-water during malting to reduce the occurrence of spoilage organisms, e.g. Gram-negative bacteria and *Fusarium* spp. (Linko *et al.* 1998).

In this study we isolated sixty-seven different LAB and several Gram-negative bacteria strains throughout the malting process of two barley cultivars, i.e. Clipper (local cultivar) and Prisma (imported cultivar), malted at Southern Associated Maltsters (SAM), Caledon, South Africa. The LAB were identified to species level using phenotypic and genotypic methods. The effect of these bacteria on the fermenting ability of brewer's yeast was also investigated.

TAXONOMY OF BACTERIA ISOLATED FROM MALT

Little has been published on the LAB population during the various phases of commercial malting. The numbers of bacteria on barley may increase up to 700-fold during the production of green malt (O'Sullivan *et al.* 1998). The first phase of this study was to determine the number of bacteria during the malting of Clipper and Prisma barley and to identify the strains to species level. The bacteria were isolated from ten different phases of four individual runs of each cultivar. The Gram-negative bacteria isolated from Clipper and

Prisma were identified as *Citrobacter* spp., *Enterobacter* spp., *Pantoea* spp., *Proteus* spp., *Seratia* spp., *Kluyvera* spp., *Klebsiella* spp., *E. coli* and *Vibrio* spp., based on API 20E carbohydrate fermentation reactions. The sugar fermentation patterns are listed in the addendum. Sixty-seven Gram-positive bacterial colonies were selected from MRS-agar based on differences in morphology.

The cell counts of the LAB in the four individual runs of the two barley cultivars did not vary by more than 5%. After the first contact with water the LAB counts steadily increased from 1.2×10^3 to 9.0×10^5 for Clipper and from 7.6×10^4 to 5.5×10^5 for Prisma. Slightly less LAB were isolated from the barley in the first dry stand, probably due to the lower water activity in the kernels. After the second steep the cell counts increased again to numbers as high as 2.4×10^7 and 8.4×10^7, as detected in samples taken from barley after the second dry stand. Similar results have been reported in other studies (Douglas and Flannigan 1988; Peters *et al.*1988; Noots *et al.* 1999). The rapid proliferation of LAB is ascribed to the hydration of the barley kernel and the leakage of nutrients into the steep water (Noots *et al.* 1999). Kilning brought about a reduction in bacterial counts to almost the same than observed in barley before malting. This reduction is ascribed to the high temperatures during kilning (Noots *et al.* 1999).

Thirty-eight of the isolates did not produce NH_3 from arginine and produced mainly D(-)-lactic acid and CO_2 from glucose. These isolates were classified as members of the genus *Leuconostoc* based on their carbohydrate fermentation patterns. Twenty-five strains were identified as *Leuconostoc argentinum* based on their whole cell protein profile clustering ($r \geq 0.86$) with the type strain of *Le. argentinum* (ATCC 51353^T) and were confirmed with RAPD-PCR analysis and 16S rRNA sequencing analysis. The remaining 13 isolates clustered at $r \geq 0.87$ with the type strain of *Leuconostoc lactis* (DSM 20202^T) and *Le. lactis* DSM 20192 and were confirmed as *Le. lactis* by RAPD-PCR and 16S rRNA sequencing analysis. Comparison of the whole cell protein patterns of only the type strains from the latter two species have shown an even closer phenotypic relationship at 87% (Björkroth *et al.* 2000).

Seventeen strains that produced NH_3 from arginine and produced both D(-)- and L(+)-lactic acid and CO_2 from glucose were identified as *Lactobacillus* spp. by their carbohydrate fermentation patterns. However, total soluble cell protein patterns grouped thirteen of the isolates with *Weissella confusa* ATCC 646 and 10881 at a level of $r \geq 0.91$. The remaining four strains formed a tight grouping ($r \geq 0.96$) with *Weissella paramesenteroides* ATCC 33313. 16S rRNA sequencing analysis confirmed the classification of the strains as *W. confusa* and *W. paramesenteroides*, respectively. This result confirms that carbohydrate fermentation patterns can not be solely used to classify LAB.

Seven rod-shaped strains differed from the other strains based on their sugar fermentation profiles and their inability to produce CO_2 from glucose. These strains were identified as *Lactobacillus rhamnosus* (two strains) and *Lactobacillus casei* (five strains), since they grouped into two tight protein profile clusters ($r \geq 0.95$ and $r \geq 0.96$, respectively). These two species are phenotypically closely related ($r \geq 0.89$), confirming our previous findings (Dicks et al. 1996). Five strains preliminary identified as members of the genus *Lactococcus* grouped in a tight cluster at $r \geq 0.95$ with *Lactococcus lactis* IL 1403 and were not subjected to RAPD-PCR and 16S rRNA sequencing.

FERMENTABILITY STUDIES

Microorganisms, especially bacteria, often produce metabolites which may render beer organoleptically unacceptable (Hough et al., 1982; Martens et al., 1997). These microorganisms cause turbidity, acid formation and production of off-flavours (Hough et al., 1982). The acids may induce premature yeast flocculation, interfere with yeast metabolism and many are flavour-active (Evans et al., 1999). Some strains produce extracellular slime, which gives rise to 'ropey' beer.

The second phase of this study was to determine what effect the bacteria, isolated from malting of Clipper and Prisma barley, have on the fermenting ability of a brewer's yeast strain (*Saccharomyces cerevisiae* SAB 05) in sweet wort prepared from malt of these two cultivars. The presence of both the LAB and the Gram-negative bacteria had no effect on the

yeast to reduce the gravity of the fermenting wort. As expected, the LAB caused a drop in the pH of the fermentations in both Clipper and Prisma wort due to the formation of organic acids such as lactic acid, acetic acid, decanoic acid, etc. The fermentations containing the *Leuconostoc – Lactococcus* mixture recorded the lowest end pH, while the Gram-negative combination recorded the highest end pH. The high concentrations of acetic acid detected in the fermentations containing LAB can be responsible for the low pH values recorded in the fermentations. The yeast counts in the control fermentations, in both Clipper and Prisma wort, were higher than the yeast counts in the fermentations containing the yeast and bacteria combinations, indicating that the bacteria may have a negative effect on the yeast counts. This rapid decrease in the yeast counts may be due to the decrease in pH caused by LAB, or the production of, yet unknown, antimicrobial substances by these bacteria. Another reason for the reduction in yeast counts may be due to the bacteria that metabolize some of the essential nutrients needed by the yeast. The Gram-negative bacterial counts decreased throughout the fermentation, while the LAB counts remained stable. This decrease in Gram-negative bacteria was also previously observed by various researchers who ascribed the reduction to the decrease in wort-pH to levels below 4.4 and an alcohol content above 2.0%, v/v (Gilliland and Harrison, 1966; Stratford, 1996 and Evans et al., 1999).

The overall aroma profiles recorded for the control fermentations (fermented with yeast only) in Prisma and Clipper malt were very similar, except for slightly higher levels of acetic acid and decanoic acid recorded for Prisma fermentations, suggesting that malt produced from these two cultivars share some characteristics. The fact that the Gram-negative bacteria had no significant effect on the levels of the volatile aroma compounds produced during the fermentation of Prisma and Clipper malt could be ascribed to the rapid decrease in the cell counts recorded.

The LAB had a definite effect on the aroma components produced in all the fermentations as can be seen from the decrease in pH and acetic acid production, especially after the second day of fermentation. The levels of five principle volatiles on the tenth day of the fermentations containing yeast and the different LAB genera differ from that of the control fermentations. Generally, the concentrations of these aroma compounds are lower than in

the control fermentations, suggesting that the bacteria may have an effect on the yeast in producing these metabolites. A further point of interest is the abnormal high levels of 2-phenyl ethane recorded for Prisma and Clipper malt fermented with yeast and LAB, compared to the relatively low concentrations recorded in the two control fermentations. It is tempting to speculate that these high levels of 2-phenyl ethane can also be responsible for the lower yeast counts detected in the fermentations containing LAB.

FURTHER RESEARCH

The LAB isolated from the malting of Clipper and Prisma barley were identified as *Le. lactis, Lc. lactis, Le. argentinum, W. confusa, W. paramesenteroides, Lb. casei* and *Lb. rhamnosus*. However, *W. paramesenteroides* and *Lc. lactis* were not isolated from Clipper malt, while *Le. argentinum* and *W. confusa* were not isolated from Prisma malt. The LAB population in the two barley cultivars might be different to the results obtained in this study, because only one medium (MRS) were used for isolation. Thus, using more differential media for the cultivation of LAB might give a better insight on the changing population in the malting of these two barley cultivars.

The LAB had a definite effect on the pH of fermenting wort, the yeast counts, and the level of volatile aroma compounds produced in fermentations containing yeast and LAB. Further research need to be done to identify whether the bacteria produce some of the volatile compounds produced by the yeast and thereby increase the final concentrations when yeast and bacteria are both present. The effect of different concentrations of the volatile compounds, especially acetic acid, octanoic acid, decanoic acid and 2-phenyl ethane, on the yeast cell counts and the fermenting ability of yeast during fermentation also need to be investigated. Reasons for the rapid decrease in yeast count when growing in combination with LAB also need to be studied. Possible reasons for the decrease in yeast counts might be because of a faster flocculation rate caused by the presence of the bacteria or lysis of the yeast cells due to the production of antimicrobial substances by the LAB. The bacteria may also metabolise some of the essential growth factors needed by the yeast.

REFERENCES

Aguirre, M., Collins, M.D. (1993) Lactic acid bacteria and human clinical infection. *Journal of Applied Bacteriology* **75**, 95-107.

Björkroth, K.J., Geisen, R., Schillinger, U., Weiss, N., de Vos, P., Holzapfel, W.H., Korkeala, H.J., van Vuuren, P. (2000) Characterization of *Leuconostoc gasicomitatum* sp. nov., associated with spoiled raw tomato-marinated broiler meat strips packaged under modified-atmosphere conditions. *Applied Environmental Microbiology* **66**, 3764-3772.

Dicks, L.M.T., Du Plessis, E.M., Dellaglio, F., Lauer, E. (1996) Reclassification of *Lactobacillus casei* subsp. *casei* ATCC 393 and *Lactobacillus rhamnosus* ATCC 15820 as *Lactobacillus zeae* nom. rev., designation of ATCC 334 as the neotype of *Lb. casei* subsp. *casei,* and rejection of the name *Lactobacillus paracasei. International Journal of Systematic Bacteriology* **46**, 337- 340.

De Vuyst, L., Vandamme, E.J. Antimicrobial potential of lactic acid bacteria. In *Bacteriocins of Lactic Acid Bacteria, Microbiology, Genetics and Applications*, Blackie Academic and Professional, London, 1994, 91.

Douglas, P.E., Flannigan, B. (1988) A microbial evolution in barley malt production. *Journal of the Institute of Brewing* **94**, 85-88.

Evans, D.J., Schmedding, D.J.M., Bruijnje, A., Heideman, T., King, M., Groesbeek, N.M. (1999) Flavour impact on aged beer. *Journal of the Institute of Brewing* **105**, 301-307.

Gilliland, R.B., Harrison, A.F. (1966) Flavour in beer. *Journal of Applied Bacteriology* **29**, 244-255.

Hough, J.S., Briggs, D.E., Stevens, R., Young, T.W., *Malting and Brewing Science 2nd ed.* Chapman and Hall, London, 1982, 566.

Kelly, L. and Briggs, D.E. (1992) The influence of grain microflora on the germinative physiology of barley. *Journal of the Institute of Brew*ing **98**, 395-400.

Linko, M., Haikara, A., Ritala, A., Penttila, M. (1998) Recent advantages in the malting and brewing industry. *Journal of Biotechnology* **65**, 85-98.

Martens, H., Iserentant, D. and Verachtert, H. (1997) Microbial aspects of mixed yeast-bacterial fermentation in the production of a special Belgian acidic ale. *Journal of the Institute of Brewing* **103**, 85.

Noots, I., Delcour, J.A., Michiels, W. (1999) From field barley to malt: detection and specification of microbial activity for quality aspects. *Critical Reviews in Microbiology* **25**, 121-153.

O'Sullivan, T.F., Walsh, Y. O'Mahony, A., Fitzgerald, G.F., van Sinderen, D., (1998) A comparitive study of malthouse and brewhouse microflora. *Journal of the Institute of Brewing* **105**, 55-61.

Peters, H.I., Flannigan, B., Austen, B. (1988) Quantitative and qualitative studies of the microflora of barley malt production, *Journal of Applied Bacteriology* **65**, 279-297.

Stratford, M. (1996) Induction of flocculation in brewing yeasts by change in pH value. *FEMS Microbiological Letters* **136**, 13-18.

ADDENDUM: PRELIMINARY IDENTIFICATION OF GRAM-NEGATIVE BACTERIA ISOLATED FROM BARLEY DURING THE MALTING PROCESS

PRELIMINARY IDENTIFICATION OF GRAM-NEGATIVE BACTERIA ISOLATED FROM BARLEY DURING THE MALTING PROCESS

ISOLATION

Gram-negative bacteria were isolated from ten phases throughout the malting processes of two barley cultivars, Clipper and Prisma, malted at SAM, Caledon, South Africa. The samples were taken from four individual runs of each cultivar to represent the following phases: dry barley before steep, water from the first steep water-stand, barley after draining of the first steep, water from the second steep water-stand, barley from the second steep water-stand, barley after draining of the second steep, barley from the first, second and third days of germination in the germination vessels (GV), and malt after kilning.

Barley and malt samples (approx. 1kg) were collected at seven points in the sampling vessels with a sterile cylindrical tube sampler. The steep-water samples (approx. 1L) were collected directly from the steep vessels using a sterile flask attached to a nylon string. The barley and malt samples (5g) were mashed in a warring blender (Warring Commercial), after which 1g was suspended in 9ml sterile distilled water and serially diluted. The steep water samples were also serially diluted in 9ml sterile distilled water.

The bacteria were isolated by spread-plating 100µl of each dilution onto Nutrient Agar (Diagnostics Pasteur), Brilliant Green agar (Biolab) and Plate Count agar (Biolab). Incubation was at 37°C for 24h.

IDENTIFICATION

Colonies of different morphology were selected from the MRS agar plates and streaked out for pure cultures on the same medium. Gram stains were conducted on pure cultures. Carbohydrate fermentation reactions were recorded by using the API 20E system of bioMerieux (Marcy l'Etoile, France). The results of the carbohydrate fermentations are shown in Tabel 1.

RESULTS

The Gram-negative bacteria were identified as:

1. *Citrobacter* spp.
2. *Enterobacter* spp.
3. *Pantoea* spp.
4. *Proteus* spp.
5. *Seratia* spp.
6. *Kluyvera* spp.
7. *Klebsiella* spp.
8. *E.coli.*
9. *Vibrio* spp.

Table 1. Identification of Gram-negative bacteria based on carbohydrate fermentation reactions[a]

Isolate	ONP	ADH	LDC	ODC	CIT	H_2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA
Citrobacter spp.	+	(+)	-	+	(+)	-	-	-	+	-	-	+	+	-	+	+	-	-	+	+
Enterobacter spp	+	-	+	+	(+)	-	-	-	-	(+)	-	+	+	+	+	+	+	+	+	+
Pantoea spp.	+	-	-	+	+	-	+	-	+	-	(+)	+	-	-	-	-	-	-	-	-
Proteus spp.	-	-	-	+	(+)	+	+	+	-	-	(+)	+	-	-	-	-	-	-	-	-
Seratia spp.	+	-	-	-	+	-	-	-	-	+	+	+	+	(+)	+	+	+	+	+	+
Kluyvera spp.	+	-	-	+	(+)	-	-	-	(+)	-	-	+	+	-	-	+	-	+	+	+
Klebsiella spp.	+	-	+	-	+	-	(+)	(+)	-	+	-	-	+	+	+	+	+	+	+	+
E.coli	+	-	(+)	(+)	-	-	-	+	-	-	+	+	-	+	+	-	-	(+)	-	+
Vibrio spp.	+	-	+	+	+	-	-	-	+	-	+	+	-	-	-	-	-	+	-	(+)

[a] +, 90% or more of the strains are positive; (+), delayed positive reaction; -, 90% or more of the strains are negative; d, 11 to 98% of the strains are positive; ND, not determined.

Substrates: ONPG = ortho-nitro-phenyl-β-D-galactopyranoside, ADH = arginine, LDC = lysine, ODC = ornithine, CIT = sodium citrate, H_2S = sodium thiosulfate, URE = urea, TDA = tryptophane, IND = tryptophane, VP = sodium pyruvate, GEL = Kohn's gelatine, GLU = glucose, MAN = mannitol, INO = inositol, SOR = sorbitol, RHA = rhamnose, SAC = sucrose, MEL = melibiose, AMY = amygdalin, ARA = arabinose